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PROTEIN EXCRETION AND DROPLET FORMATION IN THE MAMMALIAN KIDNEY

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IT is generally accepted that protein is filtered by the mammalian glomerulus and then reabsorbed by the tubules (Smetana, 1947; Rather, 1952; Davies, 1954; Oliver, MacDonald and Lee, 1954). Thus proteinuria results from an excessive protein load due either to an abnormally porous glomerulus or increased plasma protein concentration, or to impaired tubular resorption of filtered protein. Although this hypothesis accords well with the clinico-pathological findings in renal disease, there is little direct experimental evidence in favour of tubular resorption of protein by the mammalian kidney. The increase in the number of protein granules which has been found in the proximal convoluted tubules of the rat kidney after intraperitoneal injections of protein has been attributed to resorption of filtered protein. (Smetana, 1947; Rather, 1952; Davies, 1954; Oliver *et al.*, 1954). The present experiments were done in order to test the converse hypothesis, namely that the granules in the cells of the proximal convoluted tubules of the rat were secretory, and the proteinuria might therefore be due to tubular secretion of protein.

MATERIALS AND METHODS

Male and female Wistar rats aged 4–8 months were anaesthetized with ether and one ureter was ligated through a central or flank incision. Not less than 3 min. later, when glomerular filtration was likely to have ceased (Malvin, Wilde and Sullivan, 1958; Pitts, Gurd, Kessler and Hierholzer, 1958), 2 ml. of a solution of albumin was injected through a femoral vein. The rats were killed 3–120 min. after this injection. In some of the experiments 5 ml. of water was injected subcutaneously to induce a diuresis in order to fill the duct system more rapidly and hence to reduce the time taken for cessation of glomerular filtration after ureteric ligation.

Albumin solutions.—(*a*) Fresh egg-white and 0·9 per cent NaCl were mixed in equal proportions and filtered through gauze. The albumin content of this solution was approximately 5 per cent.

(*b*) Crystalline egg albumin was dissolved in 0·9 per cent NaCl, and the solution centrifuged to remove insoluble material. This solution contained 30 per cent albumin. Tetracycline 2 mg./100 ml. was added and the solution was stored at − 4°.

Histology.—Kidneys were fixed in Helly's solution and formol saline. Sections were cut at 3–5 μ and stained with Heidenhain's iron haematoxylin, haematoxylin and eosin, the Periodic Acid Schiff reagent (PAS) and Gram's stain.

Accurate measurement of number and size of granules was not possible. The granules in each section were therefore assessed visually. Frequent cross-checks were made to ensure reproducibility of grading.

RESULTS

Normal rats

The granules seen in the proximal convoluted tubules of normal rat kidneys (Fig. 1*a* and 1*b*) appeared to be identical with the protein granules described by previous workers (Davies, 1954; Oliver *et al.*, 1954). They stained well with Heidenhain's iron haematoxylin which was used throughout for making comparisons. PAS and Gram staining was less intense. Granules were seen in all kidneys. There was considerable individual variation in their number and size and distribution. Female kidneys contained fewer granules and they appeared smaller and more diffuse in their distribution within the cells of the proximal convoluted tubules than in male kidneys (Fig. 1*a* and 1*b*). Both kidneys were compared from each of 12 adult male and 12 adult female rats. The number, size and distribution of granules appeared equal in both kidneys from individual rats.

The effect of intravenous egg albumin

Egg albumin (5 *per cent*).—A volume of 2 ml. of this solution was injected intravenously into 11 male and 15 female rats 3–12 min. after ligation of one ureter. The animals were killed 3–25 min. later. The granules were increased in 8 of the females and in 2 of the males as compared with normal rats of the same sex. This increase was equal in ureter-ligated and control kidney from each rat and no difference was observed in the number of granules in ureter-ligated and control kidneys from the same rat in any of the 26 rats in this group.

An increase in the number of granules was usually seen first in the proximal convoluted tubule cells which lay immediately under the capsule (Fig. 2). A water diuresis was induced in 10 of the male rats before ligation of one ureter. 2 ml. of 50 per cent egg white in 0·9 per cent NaCl was injected 90–210 min. after ligation of the ureter. Seven of these rats were killed 3–15 min. after this injection and 3 were killed 1 hr. later. The granules were increased in the 3 rats killed 1 hr. after the protein injection and in 2 of the 7 killed within 15 min. of the injection, but remained equal in both ureter-ligated and control kidneys from each rat.

Crystalline egg albumin (30 *per cent*).—A volume of 2 ml. of this solution was injected iv 8–12 min. after ligation of one ureter in 6 female and 6 male rats. The animals were killed at intervals from 5–120 min. after the injection. The number of granules was increased in 2 out of the 6 female rats and in 3 out of the 6 males (Fig. 3). Vacuolation of the cells of the proximal convoluted tubules which was observed after protein injection has been reported by previous workers. (Laas, 1932; Rather, 1952.)

Protein in capsular space and tubular lumen.—Protein was seen rarely in capsular spaces of control kidneys and in kidneys after injection of 50 per cent fresh egg white in saline (5 per cent egg albumin). By contrast there was protein in 1 in 4 to 1 in 2 capsular spaces after injection of 30 per cent crystalline egg albumin.

Some indication of the concentration of protein in the tubular lumen could be gained by the intensity of staining of the protein precipitate. Protein appeared in the tubular lumen as early as 3 min. after its injection (whether as 30 per cent crystalline albumin or 50 per cent fresh egg white). It consistently appeared first in the outermost cortical tubules. Some 3–10 min. after protein injection the protein precipitate in the lumen of the tubules of the ureter-ligated kidneys was equal to or only slightly less than that seen in the control kidneys from the same rats (Fig. 2, 3 and 4). Surprisingly protein was found in some of the collecting ducts in the medulla as soon as 3–10 min. after injection in ureter-ligated kidneys (Fig. 5). After 10 min. the lumen of the tubules became progressively more filled with protein from proximal convoluted tubule to papilla, ureter-ligated kidneys containing equal or slightly smaller amounts of protein than control kidneys (Fig. 4). This was so whether the protein was injected 3 min. or as long as 120 min. after ureteric ligation.

There was no apparent correlation between the number of granules in the cells of the proximal convoluted tubules and the protein precipitate within the lumen. This was true of control and ureter-ligated kidneys (Fig. 4).

Occasionally the protein in the lumen was granular and resembled the granules in the tubular cells (Fig. 2*a*). This may however have been a histological artefact, rather than evidence of tubular secretion.

Ureteric ligation alone.—Both kidneys were compared from 6 male and 5 female rats killed 24 hr. after unilateral ureteric ligation. A slight protein precipitate was seen in some of the tubules in the ureter-ligated kidney but there were fewer granules in 7 of the 11 ureter-ligated kidneys.

Toxicity studies.—The increase in granules after intravenous protein injections was not due to renal damage. Male and female rats were injected with 2 ml. 30 per cent crystalline egg albumin intravenously (6 rats) and 2 ml. 50 per cent fresh egg white (7 rats). There was no histological evidence of renal damage one week after these injections. The granules in the cells of the proximal convoluted tubules were increased one week after 30 per cent crystalline egg albumin but not after 50 per cent egg white (Fig. 6).

DISCUSSION

The granules which appear in the proximal convoluted tubule of the kidney and the urine which is excreted after injection of various proteins (including egg albumin) contain the injected protein. This has been shown immunologically (Lippman, 1949), histochemically (Oliver, Moses, MacDowell and Lee, 1954) and by labelling the injected protein with dyes (Smetana, 1947 ; Sellers, Griggs, Marmorston and Goodman, 1954). In the present experiments there was no evidence of renal damage up to one week after injection of egg albumin. Thus the increase in the number of granules appears to represent a physiological response to injected protein rather than an effect of injury. We have found this increase in the number of granules after intravenous injections of egg albumin to be inconstant. This was also reflected in the lack of correlation between the protein concentration within the tubular lumen and the number of granules within the cells after protein injection. It seems likely therefore that proteinuria and tubular granules have no simple quantitative relationship. This conclusion is supported by the persistence of these

granules in the kidneys of rats killed one week after intravenous injection of egg albumin.

The protein granules are thought by Smetana (1947), Rather (1952), Davies (1954) and Oliver *et al.* (1954) to be filtered protein undergoing tubular resorption, although the experiments of these workers do not in themselves provide evidence which could allow this conclusion. The early appearance of protein which these workers noted at the luminal margins of the cells cannot be accepted as evidence of direction of protein transport.

In the dog, occlusion of a ureter during osmotic diuresis is said to stop glomerular filtration in 3 min. (Malvin *et al.*, 1958 ; Pitts *et al.*, 1958). It is therefore likely that glomerular filtration was considerably reduced if not halted in the present experiments. Despite this the protein granules increased equally in ureter-occluded and control kidneys. Most of the experiments were done with kidneys which contained fewer than maximal numbers of protein granules. Thus if protein filtration had continued but at a reduced rate its tubular resorption would have resulted in fewer granules in the ureter ligated kidney than in the control kidney. The present observations can therefore only be harmonised with the filtration-resorption hypothesis if it is conceded that protein filtration in the ureter-ligated kidney was equal to that in the control kidney. Although this would appear unlikely from the results of many experiments by workers from different centres using the " stop-flow " technique (Malvin *et al.*, 1958 ; Pitts, *et al.*, 1958) and from the earlier studies of Winton (1951), it cannot be excluded. Indeed, the remarkably rapid passage of protein down the tubule in the ureter-ligated kidney is suggestive

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EXPLANATION OF PLATES

FIG. 1.—Normal rat kidneys ×200. Heidenhain's iron haematoxylin.
(*a*) Male. (*b*) Female.
There are few granules and no protein in the lumen.

FIG. 2.—Right and left kidneys from a female rat (×200). Heidenhain's iron haematoxylin. The left ureter was ligated and 4 min later 2 ml. of 5 per cent egg albumin (50 per cent egg white) was injected iv. Both kidneys were excised 4 min. after this injection.

(*a*) Right kidney (control). (*b*) Left kidney (ureter-ligated).

There is a moderate increase in the number of protein granules compared with the normal female and there is a protein precipitate in the lumen of some of the tubules of both kidneys.

FIG. 3.—Right and left kidneys from a male rat (×200). Heidenhain's iron haematoxylin. The right ureter was ligated and 7 min. later 2 ml. of 30 per cent egg albumin was injected iv. Both kidneys were excised 60 min. after this injection.

(*a*) Left kidney (control). (*b*) Right kidney (ureter-ligated).

There is a considerable increase in the number of protein granules in both kidneys and there is a protein precipitate in the lumen of the tubules.

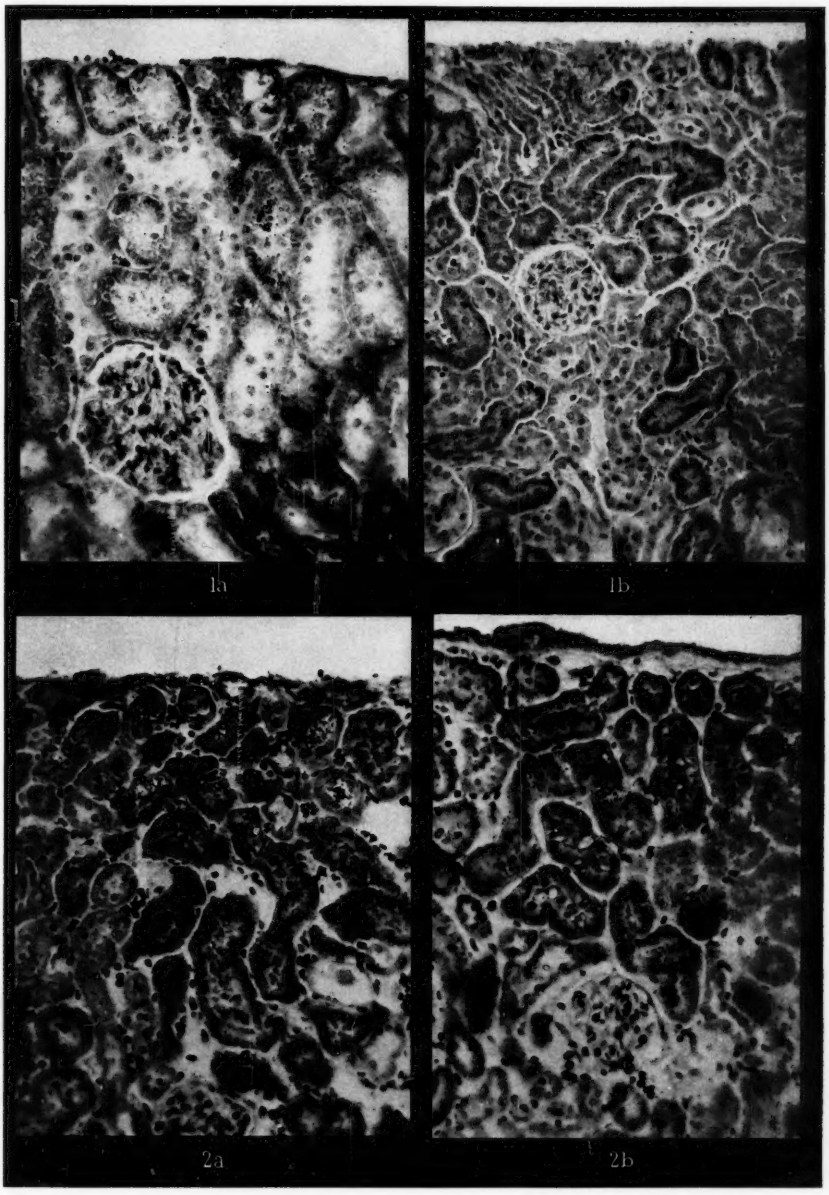
FIG. 4.—Right and left kidneys from a female rat (×200). Heidenhain's iron haematoxylin. The left ureter was ligated and 15 min. later 2 ml. of 30 per cent egg albumin was injected iv. Both kidneys were excised 60 min. after this injection.

(*a*) Right kidney (control). (*b*) Left kidney (ureter ligated).

There is a dense protein precipitate in the lumen of many of the tubules. There is some vacuolation of the tubule cells but only scanty protein granules.

FIG. 5.—Papilla of kidney shown in Fig. 2*b* (×38) to show protein in the collecting tubules of a ureter-ligated kidney 4 min after intravenous injection of egg albumin.

FIG. 6.—Kidney of a male rat (×300, Heidenhain's iron haematoxylin) one week after 2 ml. 30 per cent egg albumin iv. to show persistence of granules.



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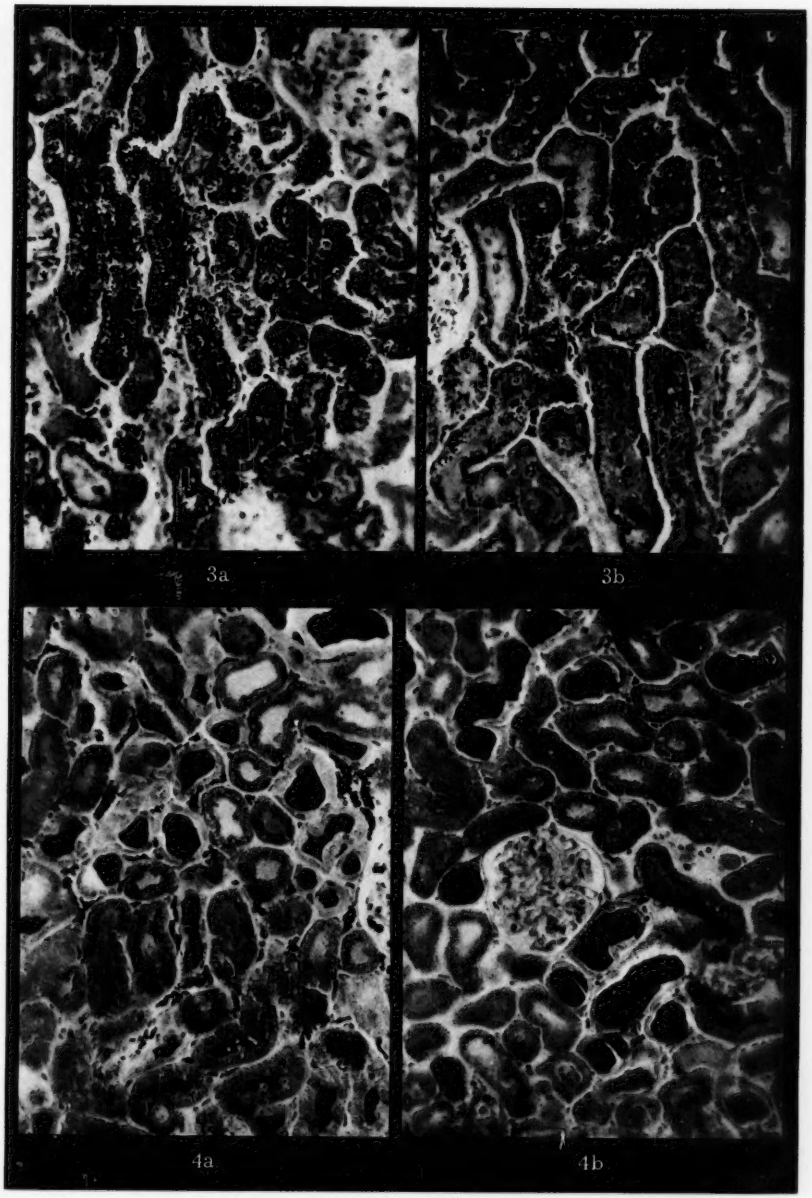

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4a 4b

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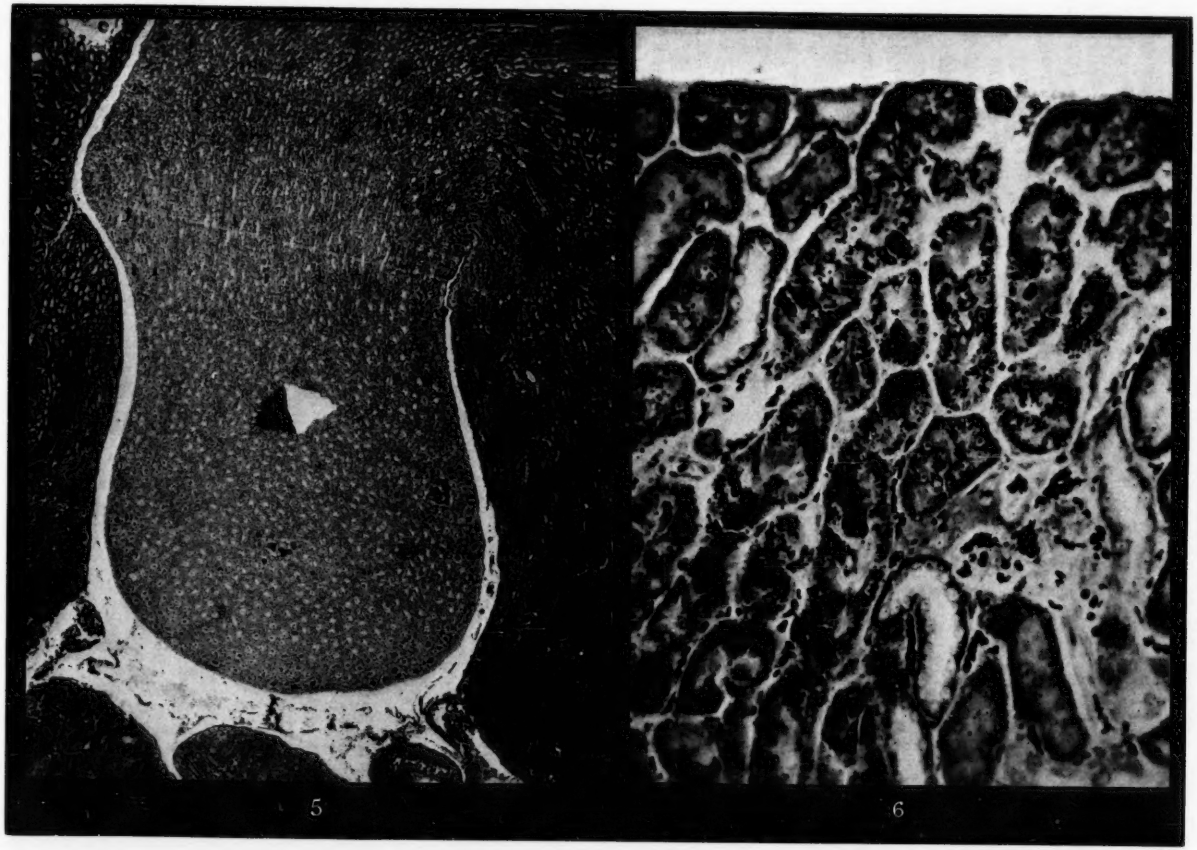

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of some flow of tubular fluid from the proximal tubule to the papilla. Thus the present experiments may be interpreted in two ways depending upon function in the ureter-occluded kidney. If, when a ureter is obstructed, filtration stops and " an essentially stationary column of fluid remains in contact with the tubular epithelium " (Pitts *et al.*, 1958), then the protein granules in the rat kidney are secretory. Alternatively, it may be that the tubular fluid is not static but in a state of dynamic equilibrium with continued diffusion across the glomeruli and tubular reabsorption. The results of " stop-flow " experiments would then represent a static cross-section of this dynamic equilibrium. If, nevertheless, the present understanding of function in ureter-occluded kidneys is correct, it appears likely that secretion of protein occurs in the rat kidney. There is no evidence at present which would allow of extension of this conclusion to other species. Indeed there is evidence to the contrary in the fish, since proteinuria does not occur in the aglomerular fish *Opsanus tau* (Beiter, 1931) and in the frog and salamander (Gerard, 1936 ; Lison, 1937). It is, however, of interest that Bence-Jones' and similar proteins are not found in the blood of patients excreting large quantities of this protein in the urine (Cross, 1957). Bence Jones' protein may therefore be secreted by the renal tubule cells, possibly after breakdown of a precursor

SUMMARY

Volumes of 2 ml. of 5 per cent egg albumin (50 per cent fresh egg white) or of 30 per cent egg albumin were injected intravenously into rats 3–210 min. after ligation of one ureter. The rats were killed 3–120 min. later. There was an increase in the number of protein granules in the proximal convoluted tubules of some of the rats compared with the normal. This increase was equal in ureter-ligated and control kidneys from the same rat.

There was no correlation between the number of granules in the proximal convoluted tubule cells and the density of the protein precipitate in the tubular lumen.

As soon as 3–10 min. after the albumin was injected protein appeared in the collecting ducts as far as the papilla.

It is concluded that (*a*) There is evidence of continued flow of fluid within the lumen of the ureter-ligated kidney. (*b*) If, nevertheless, as is currently believed, glomerular filtration stops in the ureter-ligated kidney, then protein secretion occurs in the mammalian kidney. (*c*) Proteinuria and granule formation have no simple quantitative relationship to one another.

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SOME PROPERTIES OF HUMAN ANTI-ANTIBODY

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In 1956, Milgrom, Dubiski and Wozniczko reported the demonstration, in certain human sera, of a factor capable of agglutinating human group O Rh +ve erythrocytes which had been previously sensitized with an incomplete anti-D serum. Since this factor did not agglutinate non-sensitized Rh +ve erythrocytes, they concluded that it reacted with the anti-D γ globulin attached to the erythrocytes and applied to it the term anti-antibody. The serum factor differed from Coombs anti-globulin serum in that its agglutinating property was not inhibited by the presence of free γ globulin. Erythrocytes coated with β globulin, such as naturally occurring anti-B did not absorb the anti-antibody, and natural iso-antibody which had been eluted from erythrocytes did not annul the anti-antibody whereas eluted anti-D γ globulin did so.

As eluted anti-D γ globulin neutralized anti-antibody while neither unaltered nor heat inactivated anti-D did so, these authors concluded that immunological combination of an antibody could alter its γ globulin so that it was antigenically distinct from normal or heat denatured γ globulin.

Further examples of naturally occurring human anti-antibody have been described by Grubb (1956), Lille-Szyszkowicz and Gulmantowicz (1958), Ropartz, Lenoir, Hurel, Hemet and Breteau (1958*a*) and Unger, Wiener and Katz (1958).

This paper describes the incidence and some of the more important properties of naturally occurring human anti-antibody.

MATERIAL AND METHODS

Human sera.—All sera were stored at − 16°. Five different human incomplete anti-D sera (all of titre 1 : 1000 in albumin) were used for sensitization of erythrocytes. Sera were tested from 1154 blood donors and 1471 hospital patients.

Erythrocytes.—Group O Rh +ve erythrocytes from a single donor were used in the test. In the course of the investigation it became apparent that stored erythrocytes were unsuitable and subsequently only freshly withdrawn erythrocytes were used.

Method of detection of human anti-antibody.—A 2 per cent suspension of erythrocytes in a 1 : 4 saline dilution of an incomplete anti-D serum was incubated at 37° for 30 min. The erythrocytes were then washed with normal saline three times and reconstituted as a 2 per cent suspension in normal saline. Sera were screened for anti-antibody activity at a dilution of 1 : 4 in normal saline by incubation with an equal volume (1 drop) of sensitized erythrocyte suspension at 37° for 1 hr. Any serum producing agglutination in this screening test was then titrated in doubling dilutions. Only those sera showing a titre of 1 : 8 or higher were considered positive. Control tests using unsensitized erythrocytes and unwashed sensitized erythrocytes were performed with each positive serum to exclude the possibility of false positive results due to the presence of haemagglutinin in the first case and of iso-antibodies to the Gm^a and Gm^b systems in the second.

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Dialysis.—Sera were dialysed in 24/32 in. Visking dialysis tubing, against normal saline. The dialysing fluid was changed repeatedly so that the membrane was exposed to about 20 l. over a period of 3 days.

Electrophoresis.—Sera were electrophoresed on Whatman No. 3 filter paper using a barbitone buffer of ionic strength 0·1 and pH 8·6 and a current of 0·4 ma per cm. width. The strips were then cut longitudinally and one half was stained with bromophenol blue while the other was cut transversely into segments. Each of these segments was extracted with 2 drops of a 1 : 16 dilution of normal serum in saline and the extracts were titrated in the usual manner.

RESULTS

Incidence of human anti-antibody

This was studied in two populations: Blood donors from the West of Scotland and patients whose blood was sent for cross-matching at The Haematology Department, Western Infirmary, Glasgow.

Two surveys were undertaken. In the first, the erythrocytes were of various ages and supplied by several donors, while in the second only fresh erythrocytes

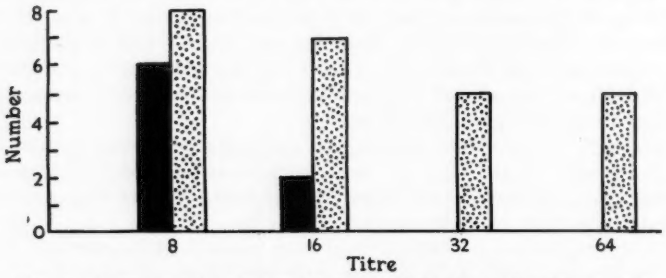


FIG. 1.—Frequency distribution histogram of the titres of positive sera in (*a*) blood donors (solid); (*b*) hospital patients (dotted).

from a single donor were used. The results are shown in Table I. In both surveys, the incidence was much higher in the hospital patients, but the table shows also that the detection rate is higher in the second survey, emphasizing that many cases will be missed unless fresh erythrocytes are used for each test. Fig. 1 shows the frequency distribution histograms of the titres of the positive sera in the 2 populations when tested with fresh erythrocytes and demonstrates that titres higher than 1 : 16 were detected only in hospital patients.

TABLE I.—*Incidence of Human Anti-antibody*

	Fresh erythrocytes	Random age erythrocytes
Blood donors	4 in 490 (0·82 per cent)	1 in 828 (0·12 per cent)
Hospital patients	25 in 664 (3·76 per cent)	8 in 643 (1·24 per cent)

Properties of human anti-antibody

Stability.—Sera showing anti-antibody did not show any change in titre during storage over a period of 8 months at −16°.

Thermal range.—Identical titres were obtained when titrations of anti-antibody were incubated at 4°, 16° and 37°. Subsequently titrations were performed at 37° to avoid confusion by the presence of cold agglutinins in the test sera.

Effect of heat inactivation at 56° for 30 min.—Heat-inactivated anti-antibody sera were titrated with erythrocytes which had been sensitized by heat-inactivated incomplete anti-D serum. Their titres were identical with those obtained with the fresh unheated anti-antibody sera, so that the heat-labile fractions of complement do not appear to be necessary for the reaction.

Complement fixation

Tests for complement fixation were performed by incubation of dilutions of anti-antibody serum with equal volumes of 2 per cent sensitized erythrocytes and 2 MHD of guinea-pig complement at 37° for 1 hr. The erythrocytes were removed

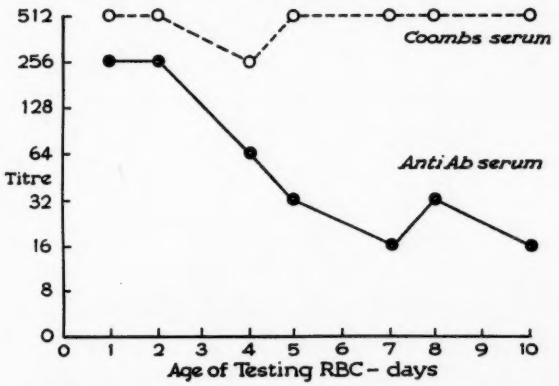


FIG. 2.—Effect of storage of erythrocytes in Rous Turner fluid on titres demonstrated by (*a*) anti-antibody ; (*b*) Coombs reagent.

by centrifugation and the supernatants added to the usual haemolytic indicator system. No evidence of complement fixation was demonstrated.

Age of testing erythrocytes.—In the preliminary experiments discrepancies were noted from day to day in the titres shown by anti-antibody sera. The erythrocytes used at this time were obtained from various donors and stored in Rous Turner fluid at 4° before use, so that the variations in results could have been explained by difference in age or genotype of erythrocytes.

In order to investigate the effect of ageing of the erythrocytes, they were stored in Rous Turner fluid at 4° for various periods up to 10 days. The cells were washed 3 times in normal saline before use, sensitized in the usual manner and rewashed 3 times with normal saline. Human anti-antibody sera and rabbit anti-human globulin (Coombs) sera were then titrated using these sensitized erythrocytes of various ages. Fig. 2 shows the marked fall in titre demonstrated in an anti-antibody serum when the erythrocytes were stored for more than 2 or 3 days, whereas the Coombs serum titre was not affected by periods of storage of cells up to 10 days. Since this ageing effect was noticed, freshly withdrawn erythrocytes have been used for each test.

Genotype of testing erythrocytes.—Fresh erythrocytes from a panel of donors were sensitized in the usual manner and used in the titration of anti-antibody sera. There were no quantitative differences in the titres demonstrated using sensitized homozygous and heterozygous erythrocytes. Thus, provided the erythrocytes possess the D antigen, the genotype does not influence the antigenicity of the sensitized erythrocytes.

pH.—Titrations were performed in iso-osmotic phosphate buffer (Hendry, 1948). Fig. 3 shows that the optimum pH range is 7·5 to 8·0. The titre falls off markedly when the pH is less than 6·5, while the anti-antibody activity is irreversibly lost by lowering the pH of the serum to 5·5 for 8 hr.

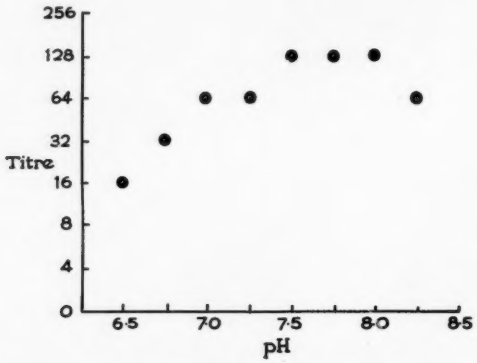


FIG. 3.—Effect of pH on anti-antibody. pH reaction carried out in isotonic phosphate buffer.

Effect of prolonged dialysis of anti-antibody serum, against normal saline.—The results of the dialysis experiments on one anti-antibody serum (serum Mu) are shown in Table II. Similar results were obtained with 2 other sera (C and Ma).

TABLE II.—*Effect of Prolonged Dialysis Against Normal Saline on Anti-antibody Serum (Serum Mu)*

	Titre
Untreated serum	1/128
Dialysed serum	1/16
Dialysed serum + 1/16 Normal serum	1/128
Dialysed serum + 1/16 Dialysed normal serum	1/32
Dialysed serum + Normal serum dialysate	1/32
Untreated serum + 1/16 Normal serum	1/128

The original titre of serum Mu was 1 : 128, but it fell to 1 : 16 after prolonged dialysis although the pH was unaltered. The titre of the dialysed anti-antibody serum was enhanced to its original level by the addition of normal human serum, which produced the full enhancing effect even when it was diluted 1 : 64 ; normal human serum showed partial enhancing activity at 1 : 128 but was inactive at 1 : 256. Normal human serum dialysed in the same manner as the anti-antibody serum did not show the enhancing effect. When normal human serum was partitioned against an equal volume of normal saline, the retentate showed the enhanc-

ing effect on dialysed anti-antibody serum, whereas the dialysate was inactive and would not restore the enhancing activity to fully dialysed normal serum. A synthetic electrolyte solution containing the major electrolytes of serum in their approximate physiological concentrations did not show the enhancing activity. It thus appears that the anti-antibody activity is enhanced by a non-dialysable co-factor which is present in normal human serum but which is destroyed on prolonged dialysis.

All the stronger anti-antibody sera detected in this survey were titrated in 1 : 16 normal serum, but none showed enhancement of titre. It was therefore concluded that the titres of these sera were not limited by their content of co-factor.

Further experiments have shown that the co-factor is not destroyed by heat inactivation or lowering of the pH to 5·5 for 8 hr. It is not affected by the removal of calcium and magnesium ions by the addition of equal volumes of 1 per cent sodium versenate or 3·8 per cent sodium citrate. The augmenting effect of normal serum is not altered by removal of the C′3 component of complement by the action of zymosan by the method described by Turk (1959) or of C′4 by the action of ammonia or hydrazine by the method described by Pillemer, Seifter and Ecker (1941).

Electrophoretic mobility.—Fig. 4 shows the results of titration of anti-antibody activity performed upon the eluates from segments of the electrophoretic strips of normal serum and serum Mu. This shows conclusively that the anti-antibody migrates with the γ globulin. Euglobulin precipitated from anti-antibody sera by the addition of distilled water was also shown to contain all the anti-antibody activity.

Specificity of human anti-antibody.—Absorption experiments were undertaken in which an anti-antibody serum was incubated at 37° for 1 hr. with the immune complexes formed with human antibodies. The immune complexes were then removed by centrifugation and the anti-antibody activity of the supernatant sera was titrated. The immune complexes used were : (1) Precipitate formed by human thyroid extract and Hashimoto disease serum. (2) Killed *Salmonella typhi* agglutinated by serum of patients convalescing from typhoid fever. (3) Killed *Brucella abortus* agglutinated by the serum of convalescent brucellosis cases.

Incubation with these antigen-antibody complexes did not reduce the titre of the anti-antibody serum.

DISCUSSION

Incidence of naturally occurring human anti-antibody

A recent annotation in the Lancet (1960) discusses the limitation of a survey of blood donor sera as a method of sampling the population and points out that there will be no children in the sample, while the Registrar General's classes I and II will be over-represented. Marks and Coombs (1957) have also emphasized that though blood donors are usually free from gross disease, some may be suffering from or have recently recovered from minor infections.

A survey of blood donors was undertaken despite these objections, as it was the only practical approach possible and likely to yield results approximating fairly closely to the true incidence in the population. The present series shows an incidence of 0·8 per cent in the West of Scotland. This is comparable with 0·5 per cent found in Poland (Milgrom *et al.*, 1956), 0·33 per cent in France (Ropartz *et al.*,

1958*a*) and 0·6 per cent in U.S.A. (Unger *et al.*, 1958). In the accounts of these surveys the duration of storage of the erythrocytes used in the tests is not stated, and the somewhat lower incidences reported might be explained by the effect of storage of the erythrocytes.

Anti-antibody is more common in the sera of hospital patients than in the general population. The present survey shows an incidence of 3·7 per cent in potential transfusion recipients at the Western Infirmary, Glasgow. The only previous survey of a population of this type was made by Unger *et al.* (1958) in the U.S.A. when they noted an incidence of 5·5 per cent. The figures are not, however, directly comparable as these authors accepted titres of 1 : 2 as positive while in the present survey I accepted only titres of 1 : 8 or greater as positive. It is of interest that Ropartz, Hemet, Hurel and Lenoir (1958*b*) found an incidence of 11 per cent in patients with pulmonary tuberculosis and preliminary results (Beck and Lees unpublished) show an incidence of 13 per cent in patients with this disease in Glasgow.

As far as can be determined from analysis of the patients in the present series there is no other disease in which the incidence of anti-antibody is outstandingly high. Only 2 of the positive sera were from patients who had had a previous blood transfusion; no irregular haemagglutinins active at 37° were detected while none of the patients showed evidence of active tuberculosis.

Sera were also studied in a group of patients suffering from diseases which may be auto-immune in origin. These included Hashimoto's disease, rheumatoid arthritis, disseminated lupus erythematosus, acrosclerosis, polyarteritis nodosa and cirrhosis of the liver. In a series of 100 of these cases, only one weak positive was detected, a lower incidence than in the potential transfusion recipients.

The significance of the raised incidence and higher titres of anti-antibody in the sera of diseased persons, and in particular in pulmonary tuberculosis, is not yet apparent, although the low incidence in the " auto-immune " disease group suggests that its development is not a feature of increased antibody production.

Nature of the agglutinating factor

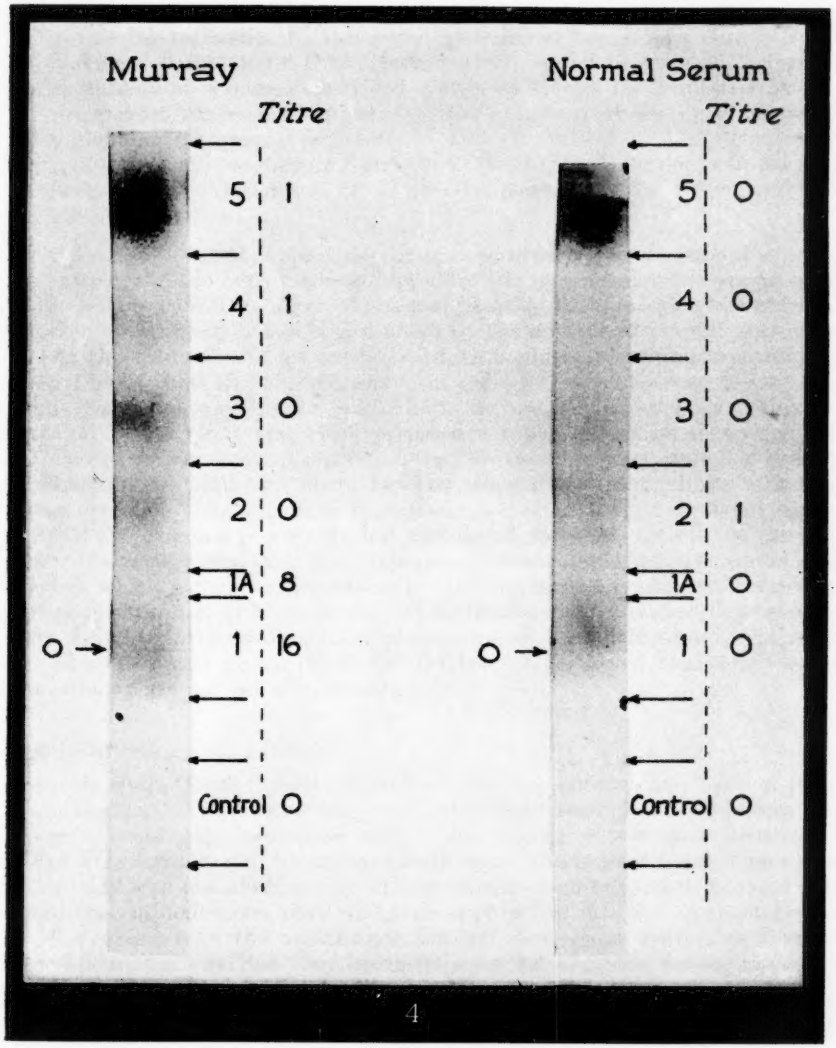
The experiments described above appear to support the hypothesis of Milgrom *et al.* (1956) that the agglutination of sensitized erythrocytes is caused by an antibody (so-called anti-antibody) specific for another antibody which has been altered by union with its appropriate antigen.

There are, however, several other possible explanations which might account for the agglutinating property of certain sera upon sensitized erythrocytes. The following possibilities have been considered and, for the reasons given below, are not regarded as supplying the correct explanation.

(1) Physical influence of colloids in the sera: this effect could occur with a serum diluted 1 : 2 or even 1 : 4 but it is unlikely to operate when the serum is diluted 1 : 8. It was for this reason that Milgrom *et al.* (1956) and I have taken 1 : 8 as the lowest titre considered positive in anti-antibody titration.

EXPLANATION OF PLATE

FIG. 4.—Electrophoretic mobility of anti-antibody.


Murray
Titre
5 1
4 1
3 0
2 0
1A 8
1 16
Control 0
O
Normal Serum
Titre
5 0
4 0
3 0
2 1
1A 0
1 0
Control 0
O
4

(2) Haemagglutinins in the serum cannot be responsible as a serum was only considered positive if controls using unsensitized erythrocytes were not agglutinated.

(3) Iso-antibodies to the serum globulin of the groups Gm^a (Grubb and Laurell, 1956) and Gm^b (Harboe, 1959) cannot be responsible for the agglutination reactions described above, as it was not inhibited by the presence of free incomplete anti-D serum in my experiments nor by pooled γ globulins (Milgrom *et al.*, 1956).

(4) Immunoconglutinin (Marks and Coombs, 1957) cannot be implicated here as heat inactivation of all the components in the system did not inhibit agglutination.

As these results would support the natural occurrence of anti-antibody in man, several attempts have been made to reproduce the phenomenon in experimental animals. Milgrom and Dubiski (1957) have shown that immunization of rabbits by escherichia and proteus bacilli agglutinated by the appropriate rabbit antiserum provoked the formation of an antibody which agglutinated sheep erythrocytes sensitized by a rabbit anti-sheep haemolysin and which was absorbed only by the immune complexes used in its production. In another series of experiments Najjar and Fisher (1955 and 1956) prepared a rabbit precipitin to an enzyme (yeast alcohol dehydrogenase) and found that the immune precipitate had greatly reduced enzymatic activity; when, however, they immunized rabbits with this immune complex, the antiserum completely inhibited the residual enzymatic activity of the immune precipitate but had no effect on the activity of the free enzyme. It was suggested from these experiments that rabbits had in fact produced antibodies which would react specifically with an immune complex formed by homologous antiserum with its antigen. These results could, however, be explained equally well by the development of iso-antibodies to rabbit serum groups as described by Dray and Young (1958) and Dubiski, Dudziak and Skalba (1959) but this possibility has not yet been investigated.

Specificity of human anti-antibody

Milgrom *et al.*, (1956) reported from their own experiments and those of Race and von Loghem that erythrocytes sensitized with all incomplete anti-D sera and some sera containing incomplete anti-C, incomplete anti-K and incomplete anti-Fy^a were agglutinated by anti-antibody sera. The present paper shows that anti-antibody was not absorbed by human anti-thyroglobulin, anti-typhoid and anti-brucella antibodies combined with their appropriate antigens. It thus appears that after union with the appropriate antigen, only some antibodies become modified in such a way that they become themselves antigenic for the naturally occurring human anti-antibody described in this paper.

Co-factor

The presence of a co-factor which augments anti-antibody and which is destroyed by prolonged dialysis against normal saline has not been described previously. This co-factor is present in normal serum which shows the augmenting effect even at high dilutions (1 : 128). The nature of this co-factor has not yet been determined but it differs from the co-factor in normal serum (Boorman and

Dodd, 1947), which enhances the titres of complete immuno-agglutinins, as this latter factor is active only in relatively high serum concentrations (1 : 3).

SUMMARY

Certain human sera, which do not contain irregular haemagglutinins, are shown to be capable of agglutinating group O Rh +ve erythrocytes which have been sensitized by a strong incomplete anti-D serum, even in the presence of excess of sensitizing serum. This property conforms to the characteristics of anti-antibody as described by Milgrom *et al.* (1956).

Anti-antibody is present in 0·8 per cent of blood donors and in 3·7 per cent of a series of hospital patients. The raised incidence in hospital patients cannot yet be explained.

Naturally occurring anti-antibody is heat stable, has an optimum pH range of 7·5–8·0 and does not fix complement in its action. It migrates with the γ globulin on electrophoresis. Fresh erythrocytes must be used in its detection as stored cells " age " rapidly for the test system.

The action of anti-antibody is diminished by removal of a previously undescribed dialysis-labile co-factor which is also present in normal human serum and is active at high dilutions.

I am deeply indebted to Professor D. F. Cappell, Dr. J. R. Anderson and Miss K. Gray for encouragement, advice and assistance throughout this investigation. Dr. J. Wallace of the West of Scotland Blood Transfusion Service kindly supplied the high titre incomplete anti-D sera and the sera from blood donors, while Dr. H. E. Hutchison of the Haematology Department of the Western Infirmary, Glasgow supplied the sera from hospital patients. My thanks are also due to Mr. R. Callendar who prepared the diagrams.

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HYPERCHOLESTEROLAEMIA, CORONARY LIPIDOSIS AND THE TISSUE MAST CELL

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WORK described in a previous study (Watson, 1958) did not support the idea that tissue mast cell depletion promotes hyperlipaemia or atherosclerosis. However, it did not examine the possibility that if mast cell deficiency really occurs in association with these states it might be secondary to them and not their cause. Grunbaum, Geary, Grande, Anderson and Glick (1957) found no difference in the mast cell count of the rat ear when hyperlipaemia had been produced by a diet of butter fat, cholesterol and thiouracil. But they did not achieve particularly high serum cholesterol levels nor, it seems, did they produce vascular lipid infiltration. Shoulders and Meng (1957), on the other hand, claimed that the intraperitoneal injection of fat emulsions caused disruption of mesenteric mast cells. However, this was probably due to the non-ionic detergents present in the emulsifying medium and not to the fat.

This study had two main aims, firstly to determine to what extent hypercholesterolaemia and coronary lipidosis affected the myocardial mast cell count, and secondly to investigate the effect of total body depletion of mast cells on the ability of the rat to correct an experimentally produced hyperlipaemia.

Three separate experiments were carried out and these are now described.

EXPERIMENT I

Materials and methods

Forty, 3-month, male albino rats were divided into 5 groups (K, L, M, N and O) of 8. Weight was distributed comparably in each group. The rats were housed separately and fed as follows.

Group K.—Normal bran. Rats, 1, 3, 5 and 7 were killed after 8 weeks and the others after 16.

Group L.—Bran, 10 per cent corn oil, 2 per cent cholesterol, 2 per cent sodium cholate, and 0·3 per cent methylthiouracil for 8 weeks.

Group M.—Bran, 2 per cent cholesterol, 2 per cent sodium cholate and 0·3 per cent methylthiouracil for 8 weeks.

Group N.—Same diet as group M, for 16 weeks.

Group O.—Same diet as group M, for 8 weeks and then normal bran for a further 8 weeks.

Water intake was unlimited, but food was restricted to 20 g. per day. The animals were weighed at the start and finish of the experiment. At the end of each dietary programme blood for cholesterol estimation by Henly's modification (1957) of the method of Zlatkis, Zak and Boyle (1953), was obtained by cardiac puncture and the animals killed with ether. The liver and kidneys were inspected and segments taken for section. The aorta and its main branches were dissected *in situ* and examined for lesions. The external surface of the heart was inspected for macroscopic lipid infiltration. It was then divided transversely and fixed with the liver and kidney slices in 10 per cent formalin, 1 per cent calcium chloride. Frozen sections of all hearts and 2 liver and kidney specimens from each group were stained for fat with Sudan IV. Paraffin sections of the other half hearts were cut at 5 μ and stained

with toluidine blue, as described previously. Preliminary examination of normal rat liver and kidney revealed that these organs were devoid of mast cells, so that a systematic study for rat liver and kidney mast cell change was not included.

This study required a quantitative estimate of mast cell density in myocardial sections. The total mast cell count for a complete cardiac section is unsuitable for numerical comparison since sections from different hearts vary in area. The method adopted therefore was to count the number of mast cells in an entire transverse section of myocardium, measure the area of the section using a square-lined microscope graticule (Watsons Ltd.), and then calculate the mast cell density of the section as the number of cells per 100 squares. It is of course essential that the sections be of even thickness.

Results

No naked eye aortic lesions were seen in any group.

All animals in group L had severe macroscopic fatty infiltration of the liver, mottled infiltrations of the kidneys and a small white plaque of varying size, though averaging about 2 × 2 mm., at the apex of the heart. Frozen sections of the liver and kidney segments showed gross fatty infiltration and in 4 of the 8 hearts intimal and medial lipidosis of coronary vessels was present (Fig. 1). No systematic serial section cutting of hearts was undertaken, but there is a reasonable probability that coronary lipidosis was present in all 8 hearts. Coronary lipidosis is the usual arterial lesion produced in rats by the short term experimental diet (Wilgram, Hartroft and Best, 1954). This is no more than an infiltration of fat-staining material into the intima, often into the media, and sometimes through to the adventitia and periadventitious tissues. The fibrosis, ulceration and calcification of adult human atherosclerosis are not seen in this particular kind of experimental lesion. Fig. 2 shows mast cells close to a coronary artery in the heart from L 8.

Similar, but less severe degrees of fatty change were present in the rats of group M, and of a degree intermediate between L and M in group N. These gradations can be explained by the different degrees and durations of hypercholesterolaemia in the 3 groups of animals.

In group O considerable regression of tissue changes had occurred. The livers were only slightly paler than normal and no lipid infiltrations were seen in hearts or kidneys. The liver sections showed only traces of lipid staining and the hearts none.

The animals' weights, cholesterol levels and myocardial mast cell counts are given in Table I. Rat N2 died 3 days before its due time. No blood was obtained for cholesterol estimation and although the heart was sectioned and stained for mast cells, post-mortem changes had occurred and only faintly staining granular remnants were visible.

The myocardial mast cell counts for the groups L, M, N and O are each compared with group K and the significance values shown in Table I. The slight variations in the average mast cell counts for each group are obviously not significantly different from group K or from one another. But it can be seen from a study of the table that there is a bigger range between the mast cell counts of individual animals in group L, M and N. The differences are not related to cholesterol levels or degree of weight loss (for all of the animals on the lipogenic diet lost weight). Another possible association might have been that the individual myocardial mast cell counts varied according to the extent of the coronary lipid infiltrations Therefore the 23 animals in these 3 groups were divided into 2

TABLE I.—*Data for 5 Groups of Rats on Different Dietary Schedules Showing* (a) *Initial Weight (g.)*; (b) *Final Weight*, (c) *Serum Cholesterol when Killed (mg. per cent) and* (d) *Mast Cells per Unit of Area. The Mast Cell Count for Each Group is Compared with Group K and the Significance Values are Shown Alongside. CO = Corn Oil; C = Cholesterol; SC = Sodium Cholate; MT = Methyl Thiouracil*

Group	Diet	Reading	1	2	3	4	5	6	7	8	Mean	
K	Bran	*a*	275	285	280	295	295	310	275	320	292	
	1, 3, 5, 7 for 8 weeks	*b*	280	310	310	350	315	335	290	360	319	
	2, 4, 6, 8 for 16 weeks	*c*	95	70	61	89	91	88	74	73	80	
		d	23·7	25·3	22·0	20·2	25·2	27·0	25·9	17·9	23·4	
L	Bran + 10 per cent CO + 2 per cent	*a*	290	285	290	300	295	310	290	320	298	
	C + 2 per cent SC + 0·3 per cent	*b*	235	240	210	215	240	275	245	265	241	
	MT for 8 weeks	*c*	2340	1330	1140	1440	1200	1406	2030	1680	1571	$t = 1·33$
		d	23·4	27·8	15·3	10·5	22·7	11·1	26·4	21·9	19·9	$P > 0·10$
M	Bran + 2 per cent C + 2 per cent	*a*	285	285	290	295	290	325	315	315	300	
	SC + 0·3 per cent MT for 8 weeks	*b*	240	240	240	230	250	265	220	250	242	
		c	676	1080	1720	1210	773	528	176	680	855	$t = 0·90$
		d	8·7	23·9	23·8	26·9	14·9	27·8	18·7	23·8	21·1	$P > 0·25$
N	As M for 16 weeks	*a*	280	290	295	295	305	305	310	330	301	
		b	230	245	230	240	250	250	260	270	247	
		c	811	—	604	1109	1006	974	978	900	912	$t = 1·36$
		d	16·9	—	11·3	13·1	30·6	21·2	27·3	15·9	19·5	$P > 0·10$
O	As M for 8 weeks then bran for 8	*a*	280	285	295	295	295	305	305	320	298	
	weeks	*b*	300	305	300	300	300	330	310	345	311	
		c	89	59	73	95	75	61	85	46	73	$t = 0·24$
		d	22·4	21·6	28·2	22·4	21·4	23·0	22·4	23·7	23·1	$P > 0·25$

categories, 16 in which the coronary artery changes were slight or absent, and 7 in which they were severe. The mean mast cell counts for these 2 groups were 19·0 and 22·9 respectively ($t = 1{\cdot}50$; $P > 0{\cdot}10$). The difference is not significant.

The conclusions which can be drawn from this experiment are :

Groups L, M and N.—" Acute " hypercholesterolaemia and coronary lipidosis of short duration and of a moderate or severe degree neither increases nor decreases the concentration of mast cells in the myocardium. The slight difference in the mean count of groups M and N is not significant ($P > 0{\cdot}25$) and provides no evidence that prolongation of a lipogenic regime might lower the count. No morphological changes were observed in the mast cells.

Group O.—Recovery from moderately severe hyperlipaemia and minor degrees of coronary lipidosis (the results of group M represent this) allowed to continue for 8 weeks after withdrawal of the lipogenic diet, is accompanied neither by an increase nor a decrease in the myocardial mast cell count. An increase might have occurred as part of some " normalizing " mechanism, in which a mast cell lipid scavenger effect came into play ; a decrease might have followed, for example, exhaustion of the cells through utilization of their heparin.

EXPERIMENT II

The last experiment left a doubt as to whether a possible normalizing or scavenger role of the mast cells had been adequately tested, or the effect looked for at the optimum time. The coronary lipidosis in group M (the group whose serum and tissue changes probably corresponded with those of group O at the point of withdrawal of the lipogenic diet) was not gross. Again the recovery period of 2 months which elapsed might have been long enough to permit a transient change in the mast cells and their subsequent return to normal. A second experiment was therefore devised and carried out as follows.

Materials and methods

Twenty-two, 1-yr.-old, male albino rats were divided into 2 groups (P and V) of 10 and 12. P is also the control group in the experiment described after this. One of the rats in this group died during the experiment. Group P was given normal bran throughout. Group V was given bran supplemented with 10 per cent corn oil, 2 per cent cholesterol, 2 per cent sodium cholate and 0·3 per cent methylthiouracil, for 8 weeks. At the end of this time blood was withdrawn by cardiac puncture, under ether anaesthesia, for serum cholesterol estimations, and all animals allowed to recover. The 2 rats with the lowest cholesterol levels (910 and 1030 mg. per cent) were killed and their hearts, livers and kidneys examined macroscopically and microscopically as described in Experiment I. Both had gross lipid infiltra-

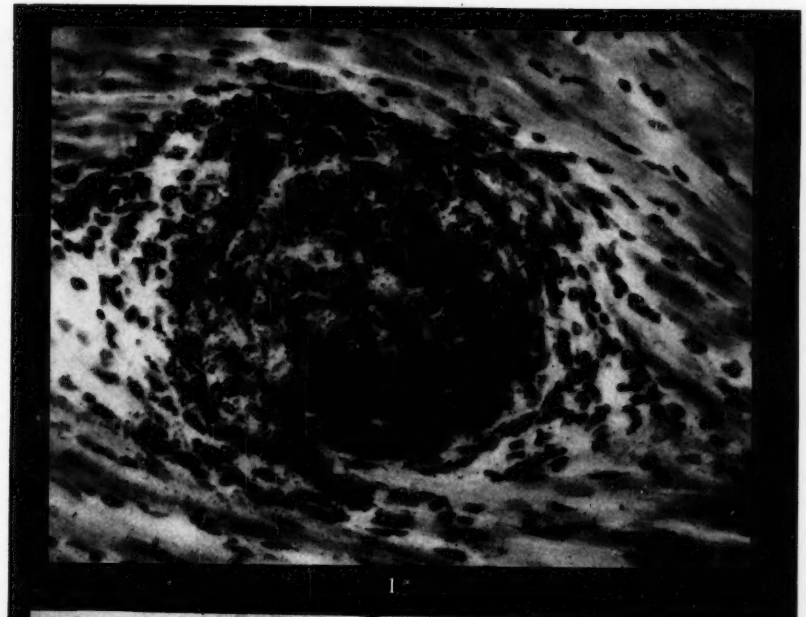
EXPLANATION OF PLATES

FIG. 1.—Frozen section of rat heart showing lipid infiltration of a coronary artery. (Sudan IV and haemalum. ×170.)

FIG. 2.—Mast cells near a coronary artery in rat heart. (Toluidine blue. ×410.)

FIG. 3.—Frozen sections of (*a*) heart (×130), (*b*) liver (×380) and (*c*) kidney (×130) showing extensive lipid infiltration of a coronary artery, liver cells, glomerular tufts and afferent arterioles. (Rat V12. Sudan IV and haemalum.)

FIG. 4.—Frozen sections showing persistence of lipidosis in (*a*) heart, (*b*) liver and (*c*) kidneys 24 days after returning to normal diet. The degree of lipid infiltration in liver and kidney is much less, however, than in Fig. 3. (Rat V11. Sudan IV and haemalum. Heart and kidney ×117 ; liver ×330.)



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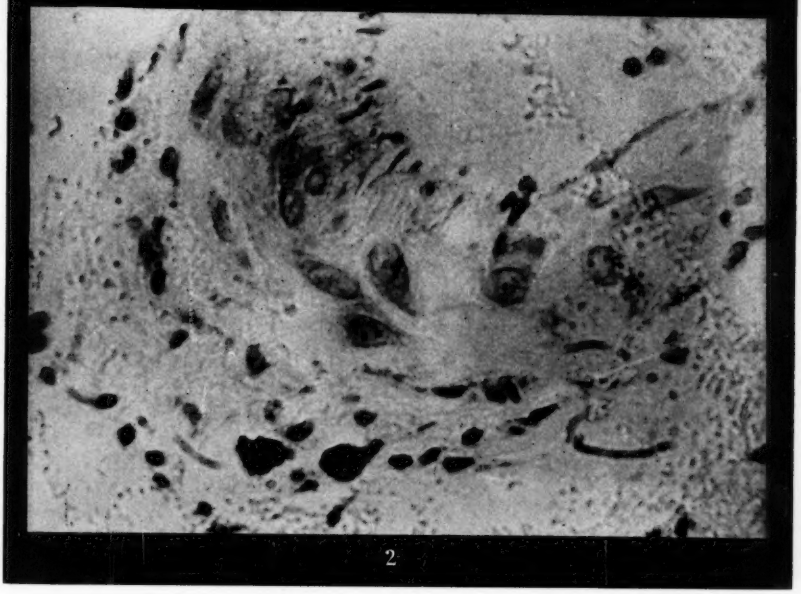

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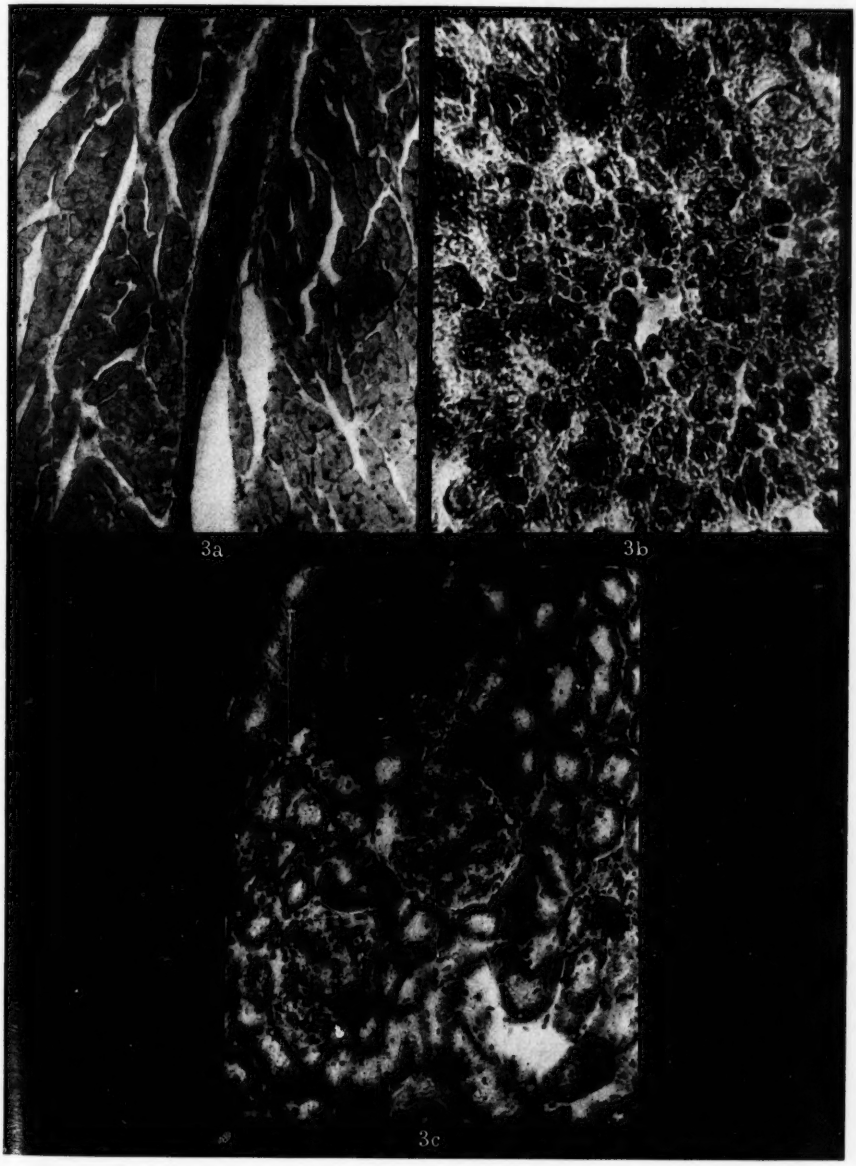
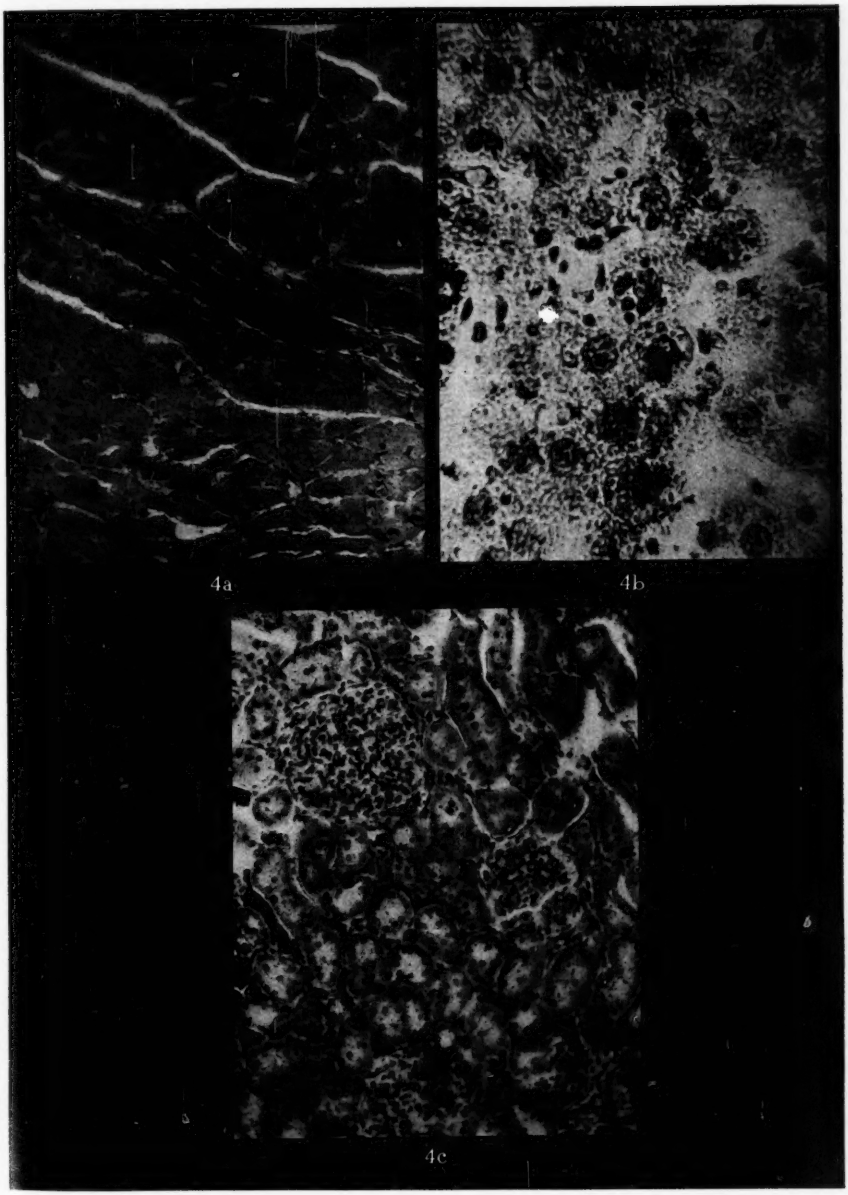
Watson.



3a 3b

3c



4a 4b

4c

tions of these organs. Fig. 3 shows sections stained for fat from heart, liver and kidney of one of them.

The remaining 10 rats, whose average serum cholesterol level at this point was 1617 mg. per cent, were returned to normal feeding for a further 24 days, at which time blood was again withdrawn and the animals killed while still anaesthetized. The organs were inspected and sectioned as already described.

Results

The serum cholesterol and myocardial mast cell counts for the 2 groups are given in Table II. It can be seen that a remarkable drop in the cholesterol levels of group V has occurred in this short time (from 1617·0 to 81·7 mg. per cent: normal average 78·2 mg. per cent). However, although the serum lipids had returned to normal the tissue lipids had not. The livers were still pale, but showing the red mottling of lipid losing areas, and in hearts and kidneys microscopic lipid deposits were present to a considerable degree (Fig. 4). The animal at the time of death was thus still in the phase of recovery from the dietetically induced organ lipidosis.

TABLE II.—*Serum Cholesterol and Myocardial Mast Cell Count of Normal Rats (Group P) and of Rats at the 24th Day of Spontaneous Recovery from Severe Hypercholesterolaemia and Coronary Lipidosis. a = Serum Cholesterol (mg. per cent) After 2 Months of Lipogenic Diet; b = Serum Cholesterol (mg. per cent) when Killed; c = Myocardial Mast Cell Count per Unit of Area*

	Group P		Group V		
No.	*b*	*c*	*a*	*b*	*c*
1	78	20·0	1280	82	12·0
2	76	23·6	1280	95	16·4
3	76	28·3	1570	80	16·2
4	82	29·5	1310	80	28·8
5	63	21·2	2340	92	14·3
6	97	19·6	1320	80	25·4
7	71	27·4	1390	90	12·4
8	69	27·5	1710	61	16·5
9	92	17·8	1600	73	10·6
11	—	—	2270	84	25·1
Mean	78·2	23·9	1617·0	81·7	17·8

The average myocardial mast cell concentration at this point of recovery was 17·8 cells/unit area. This may be significantly less than the average mast cell concentration of the control group which was 23·9/unit area ($t = 2{\cdot}32$; $0{\cdot}05 > P > 0{\cdot}02$) but it is not a convincing reduction. Nevertheless when one examines the mast cell figures for all groups of this and the previous experiment the mean figures for the 2 normal groups (K and P) come very close to each other (23·4 and 23·9, respectively) while the mean figures for the 5 groups which have been exposed to some degree of tissue lipid infiltration are to a greater or lesser degree below these values. If the figures for the two experiments are considered together the mean mast cell tissue concentration for the 17 animals of the 2 normal groups K and P is 23·7 per unit area and that for the 41 animals of groups L, M, N, O and V is 20·1 per unit area. Their mean difference of 3·6 is again in the marginal area of significance ($t = 2{\cdot}36$; $0{\cdot}05 > P > 0{\cdot}02$). The balance

of probability, however, considering the individual group statistics of Experiment I and the apparent lack of correlation between mast cell counts and degree of vascular change in individual animals, seems to be against any significant involvement of the tissue mast cell in the deposition or clearing of tissue lipid. Any claim that the greater lipid infiltration of liver and kidney reflects a vulnerability to lipid, due to the absence of mast cells, would be a superficial interpretation of the facts. For these organs by virtue of their structure, vascularity and functions are at greater risk than the myocardium.

The third and final experiment now described seems to show clearly that, so far as relatively acute changes in the rat are concerned, the tissue mast cell is not involved in tissue and blood lipid derangements.

EXPERIMENT III

Recovery from a hyperlipaemic state might occur without a demonstrable change in the number of mast cells, the cells participating in this recovery as a normal function and without such a degree of functional stress as would affect their concentration in the tissues. On the other hand if the mast cell does assist in the recovery their absence might retard it, because of impaired mobilization of tissue lipid and perhaps also because of impaired transport for excretion.

Materials and methods

The rats of Group P, as mentioned previously, acted as the control group for this experiment. Another 20 animals from the same batch, of the same age and of comparable weight, were divided into 2 groups (Q and R) of 10 each. One animal in group R died before the experiment was completed. Both groups were given a diet consisting of normal bran containing 2 per cent cholesterol, 2 per cent sodium cholate and 0·3 per cent methylthiouracil for 8 weeks. The ration was 20 g. per day and water intake was unlimited. A similar diet for a similar period had produced a mean cholesterol level of 855 mg. per cent in a younger group of rats (group M, Experiment I) and it seemed reasonable to expect a comparable increase. This expectation is largely confirmed by the rise to a mean level of 1501 mg. per cent for the 12 animals in group V whose diets contained 10 per cent corn oil in addition.

After 8 weeks of special diet both groups (Q and R) were returned to normal feeding. At this point of transition intraperitoneal injections of compound 48/80 were started in group R, the dose increasing from an initial dose of 0·1 mg. to 1 mg. over a period of 8 days. The animals showed the usual effects (Feldberg and Talesnik, 1953) but none succumbed. No intermediate histological studies were carried out to observe the progress of mast cell disruption. By this time sufficient experience of the method had been obtained to permit confidence in its effects. The injections were given on 6 days of each week.

Both groups of animals were bled and killed 28 days after the return to normal diet, the main organs inspected and sectioned as described earlier, and mesenteric spreads prepared from some of the animals in group R to confirm that the expected mast cell change had occurred.

Results

It can be seen from Table III that there is no significant difference between the cholesterol levels of the 3 groups of rats. Total tissue mast cell depletion has not prevented, although of course it may have delayed by a few days, the return to normal from high cholesterol levels. There was no difference in the degree of coronary lipidosis in the hearts of the group Q and R animals. In both groups it was either negligible or absent. The naked eye appearance of liver in both groups was comparable and microscopically there were equivalent degrees of fatty cellular infiltration.

It does not seem that the tissue mast cell is essential either for the mobilization of tissue lipids or their excretion from the blood.

TABLE III.—*Serum Cholesterol Levels of Normal Rats (Group P), Lipaemic Rats After Spontaneous Recovery (Group Q) and Lipaemic Rats After 24 Days of Compound 48/80*

	Cholesterol (mg. per cent)		
No.	P	Q	R
1	78	74	65
2	76	71	87
3	76	93	76
4	82	83	77
5	63	66	94
6	97	64	73
7	71	55	79
8	69	78	79
9	92	57	80
10	—	80	—
Mean	78·2	72·1	78·9

DISCUSSION

This work does not advance the idea that the tissue mast cell may be involved in atherosclerosis. It does not completely rebut it, for the lipid changes produced in the foregoing experiments come critically short of atherosclerosis as opposed to the mere infiltration of fat into vascular tissue, the earliest stage in the development of the atheromatous plaque. It seems clear nevertheless that this initial stage can occur in the presence of normal concentrations of mast cells and without causing their disappearance, depletion or morphological derangement.

Pollak (1957) in a careful and detailed study of mast cell incidence and distribution in the normal and diseased human vascular tree, was unable to correlate clinical and autopsy data with aortic mast cell counts. Without quoting figures he gives it as his impression that the incidence of peri-adventitial mast cells around atheromatous coronary arteries is higher than around normal vessels. The closing sentence of his account comes as a surprising *non sequitur* in an otherwise conservative and balanced discussion. He writes " The presence of mast cells in oedematous tissues and their absence in atheromatous plaques possibly implicate these cells as participants in atherogenesis ".

The ultimate experimental answer to this problem may be found in a long term study so that there is time for coronary atherosclerosis, with fibrosis or calcification, to develop. But there are some drawbacks to this, for on diets like that used here, the animals go out of condition, lose weight and have chronic diarrhoea. In addition, the thiouracil in the diet must cause profound changes in the animal's normal metabolism. After only 2 months of the diet the rat thyroid is greatly hypertrophied.

Because mast cells contain heparin and because in unphysiological doses heparin modifies lipoprotein structure there is the temptation to incriminate them in a tissue lipid defect. The mast cell presumably has some role in tissue metabolism and this, among other things, may include lipid regulation or utilization,

but on the basis of the experimental evidence produced here, the verdict for or against such a function seems to be not proven.

SUMMARY

In a previous study it has been shown that rats depleted of tissue mast cells by compound 48/80 are not unduly susceptible to the production of hypercholesterolaemia or vascular lipidosis. In this study the obverse possibilities have been investigated.

Severe hypercholesterolaemia and organ lipidosis were produced by a reliable lipogenic dietary regime. It seems clear that acute hypercholesterolaemia and coronary lipidosis have no effect on the concentration or morphology of myocardial mast cells. Recovery from such a state is not affected by the pharmacological depletion of tissue mast cells, nor in its turn does it influence the number of mast cells in intact animals.

The myocardial mast cell is probably not involved in the early stages of vascular lipid infiltration.

I am grateful to Professor L. J. Davis for his continuing encouragement in this work, to Dr. W. P. Duguid of the Royal Infirmary Institute of Pathology for some of the sections, to Messrs. Burroughs Wellcome for supplies of Compound 48/80, and to Messrs. Boots for generous supplies of dietary constituents.

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INACTIVATION OF STAPHYLOCOCCAL, TETANUS, AND DIPHTHERIA TOXINS BY GANGLIOSIDE

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WE recently reported the protective effect of the enzyme phosphatidase A against the lethal action of bacterial exotoxins in mice, and presented evidence which supported the proposition that the protection is due to the liberation *in vivo* of long chain unsaturated fatty acid and the production of lysophosphatide (North and Doery, 1958*a* and *b*, 1960 ; Doery and North, 1960).

Attention was called to Fisher's (unpublished) observation that intracerebral injection of sublethal doses of staphylococci in mice failed to produce macroscopic lesions whilst suppurative osteomyelitis of the skull often developed at the site of the needle puncture. It was suggested that the greater resistance of the brain might be related to the high concentration of phosphatides in this tissue.

Before this possibility could be examined our attention was called to Bogoch's (1957) report on the inhibition of viral haemagglutination by a mucolipid, ganglioside. Nervous tissue is rich in ganglioside and we decided to explore its effect on bacterial exotoxins *in vivo*.

Whilst this work was in progress van Heyningen (1958, 1959*a*, *b* and *c*) reported reinvestigating the fixation of tetanus toxin by the grey matter of nervous tissue. He found that a highly specific fixation occurred between tetanus toxin and mucolipid which appeared to be ganglioside. He also showed that tetanus toxin was slowly inactivated by ganglioside or a contaminant.

In our experience the inactivation of tetanus toxin by ganglioside is not specific. Experimental animals have been protected against staphylococcal and diphtheria toxin by ganglioside as well as against tetanus toxin.

MATERIALS AND METHODS

Ganglioside.—The method of preparation from ox brain was essentially that described by Bogoch (1958). Preparations which have been examined varied in percentage composition as follows : nitrogen 2·4–3·6 ; phosphorus 0·05–0·24 ; hexose (as galactose) 24–28 ; total sialic acid 24–27. Nitrogen was estimated by micro (—) Kjeldahl, phosphorus by a modification of the method of Brown (1954), hexose by the method of Hewitt (1937), sialic acid as N-acetylneuraminic acid by the method of Svennerholm (1957*b*). Aqueous solutions of gangliosides are slightly acidic with pH 5·5–5·9. One preparation (nitrogen 3·2 per cent, phosphorus 0·18 per cent, hexose 25 per cent, sialic acid 26 per cent), which was typical of others tested below, was examined in the ultracentrifuge. In a run of 1½ hr. in a field of 250,000 *g.* a 2 per cent solution in 0·2 M sodium chloride showed one peak with a leading edge, there being approximately 70 per cent of the material in the main peak.

Boiled ganglioside.—This refers to an aqueous solution of ganglioside (1 mg./ml.) which was held in a stoppered pyrex tube in a boiling water bath for 1 hr. (Since completing this paper it has been found that optimum activation is achieved by holding an aqueous solution of ganglioside for 10 min. in a waterbath at 80°).

Staphylococcal toxin.—A preparation (B624) used in previous work (North and Doery, 1960; Doery and North, 1960) was used throughout. The LD_{50} (i.v.) for mice weighing 20 g. was 1 ml. of 1 : 60 dilution.

Diphtheria toxin.—A toxic filtrate B431–4 supplied by Miss Enid Wadeson was used. The L + dose was 0·08 ml. subcut. for guinea-pigs weighing 250 g.

Tetanus toxin.—A toxic filtrate SM2132 E containing 24 Lf/ml. supplied by Mr. Adrian O'Connell was used. Diluted in 0·1 per cent gelatin saline, 0·1 ml. contained more than 2×10^7 LD_{50} s.c for mice weighing 20 g.

Experimental animals.—White mice weighing 20–22 g. were used in experiments with staphylococcal and tetanus toxins. Guinea-pigs 245–255 g. weight were used in protection tests against diphtheria toxin.

Injections.—In experiments with staphylococcal toxin and ganglioside all injections into mice, unless otherwise stated, were made i.v. Subcutaneous injections of tetanus toxin or mixtures of tetanus toxin and ganglioside were made.

In guinea-pigs all injections were made subcutaneously.

Tests for staphylococcal alpha haemolysin.—These were carried out by the method of Burnett (1931).

RESULTS

Action of ganglioside on staphylococcal toxin

All preparations of ganglioside so far prepared have been well tolerated by experimental animals. No noticeable ill effect was produced by 10 mg. of one preparation, typical of others, when injected i.v. into mice weighing 22 g.

No protection was afforded by ganglioside injected i.v. into mice shortly before they were challenged with staphylococcal toxin by the same route. However, when ganglioside and toxin were held at 37° for 1 hr. or more before injection, less than 0·1 mg. regularly protected against a dose of toxin that killed 90–100 per cent of untreated mice.

The results of a representative experiment are shown in Table I. It will be seen that after interaction with toxin for 30 min., at 37° ganglioside in the concentration used completely failed to protect (Groups 1 and 2). Actually, the mice injected with the mixture of toxin and ganglioside (Group 2) died sooner than those given toxin alone (Group 1).

TABLE I.—*Protective Effect of Ganglioside Against Action of Staphylococcal Toxin i.v. in Mice*

Group	Treatment of toxin	Day of death after challenge	Survivors 7 days
1	Toxin in saline 30 min. at 37°	1 1 1 1 1 1 1 1 2 2 2	1/12
2	,, + ganglioside 0·05 mg./ml. 30 min. at 37°	1 1 1 1 1 1 1 1 1 1 1 1	0/12
3	,, in saline 4 hr. at 37°	1 1 1 1 1 1 1 1 1 1 1 1	1/12
4	,, +ganglioside 0·05 mg./ml. 4 hr. at 37°	—	12/12

Toxin in saline (Groups 1 and 3) was incubated at 37° like toxin + ganglioside (Groups 2 and 4) before i.v. injection of 0·5 ml. into mice.

However, when incubation was continued for 4 hr., the same amount of ganglioside (0·025 mg.) gave complete protection against a dose of toxin that injected alone killed 11 out of 12 mice (Groups 3 and 4). The same pattern, increased susceptibility to the toxin after a short period of interaction and substantial protection after further incubation, has been noted repeatedly.

The results of the test shown in Table II, in which all injections were made subcutaneously, clearly indicate why ganglioside injected i.v. does not protect

against subsequent challenge with toxin. In this experiment mice (Group 1) were injected with ganglioside and toxin, the ganglioside having been previously held at 37° for 30 min. with 1 per cent serum albumin. They died just as readily as those injected with toxin alone (Group 3), whilst mice injected with toxin and ganglioside, not previously treated with albumin (Group 2), were protected.

A further point of interest is brought out in Table II. One lot of mice (Group 4) was injected s.c. with toxin and ganglioside which were drawn separately into the same syringe immediately before administration. Protection in this case contrasts with failure to protect when similar mixtures are injected i.v.

TABLE II.—*Protective Effect of Ganglioside With and Without Albumin Against Action of Staphylococcal Toxin Subcutaneously in Mice*

Group	Treatment of toxin	Day of death after challenge	Survivors 7 days
1	Toxin + ganglioside (1 mg.) in 1 per cent serum albumin	1 1 1 1 1 1 1 1 2 2	2/12
2	Toxin + ganglioside (1 mg.)	5	11/12
3	,, in saline	1 1 1 1 1 1 2 2 3	3/12
4	,, + ganglioside (1 mg.) mixed in syringe	1	11/12

Toxin + ganglioside + albumin (Group 1), toxin + ganglioside (Group 2) and toxin in saline (Group 3) were incubated at 37° for 30 min. before injecting into mice. Group 4 mice were injected with toxin and ganglioside immediately after mixing.

In other experiments a dose of ganglioside, which would otherwise protect, when added to an equal volume of 0·001 per cent human serum albumin (*i.e.* 0·01 mg./ml.) and held at 37° for 1 hr., no longer protected.

Early in these studies, solutions of ganglioside were found to be unstable. In one experiment it was found that solutions of ganglioside (0·1 mg./ml.) both in ammonium acetate buffer at pH 6·0 (0·2 M containing 0·05 M $CaCl_2$) and in saline rapidly lost potency when stored at 2°. When tested in mice against the lethal action of staphylococcal toxin, boiling was found to increase both stability and potency of ganglioside. Saline solutions of boiled ganglioside may be stored at 2° for some days without noticeable loss of potency. It was, therefore, found preferable to use heated ganglioside and the treatment arbitrarily selected was to hold in a boiling water bath for 1 hr.

Table III shows the results of one of many tests in which the potency of boiled and unheated ganglioside was compared. It will be noticed that in the concentra-

TABLE III.—*Effect of Heating Ganglioside* (100° *for* 1 *hr.*) *on its Protective Power Against Action of Staphylococcal Toxin Injected i.v. in Mice*

Group	Treatment of toxin	Time of incubation at 37° before injection	Day of death	Survivors 7 days
1	Toxin in saline	30 min.	1 1 1 1 1 1	4/10
2 M	,, + unheated ganglioside (0·1 mg./ml.)	30 min.	1 1 1 1 3	5/10
2 H	Ditto	5 hr.	—	10/10
3 M	Toxin + boiled ganglioside (0·1 mg./ml.)	30 min.	—	10/10
3 H	Ditto	5 hr.	—	10/10

Inoculum Dose 0·5 ml.

tion of ganglioside used in this test the increased potency of the boiled material is only shown when the incubation period is short. When incubated with toxin for some hours both unheated and boiled ganglioside (Groups 2H and 3H) gave complete protection.

Other tests showed, however, a minimum amount of ganglioside was required to inactivate one LD_{100} of staphylococcal toxin even when incubated for some hours. With a typical preparation this amount was approximately 0·025 mg.

The increased potency and stability of boiled ganglioside facilitated *in vivo* tests. Table IV gives the protocol of an experiment in which ganglioside was tested against approximately 12 LD_{100} of staphylococcal toxin (0·5 ml. of 1 : 2 dilution). In a test carried out 3 days beforehand 0·5 ml. of toxin diluted 1 : 24 killed 12 out of 12 mice within 48 hr. The dose of ganglioside used was 0·25 mg. per mouse. Reference to the table shows that some protective effect was obtained with ganglioside after incubation with toxin for only 15 min. (Groups 1 and 2). Ganglioside that was allowed to interact with the toxin for 4 hr. completely protected 5 out of 10 mice (Group 5). This number of survivors is in itself statistically significant without taking into consideration the fact that the first death in the Group 5 mice occurred only some hours after challenge.

TABLE IV.—*Protective Effect of Boiled Ganglioside Against Action of* 12 *Certainly Lethal Doses of Staphylococcal Toxin i.v. in Mice*

Group	Treatment of toxin	Time of death after challenge	Survivors 7 days
1	1 : 2 toxin + ganglioside injected immediately after mixing	All died within sec. of injection	0/10
2	1 : 2 toxin + ganglioside 37° 15 min.	All died within minutes	0/10
3	1 : 2 ,, ,, ,, 37° 35 ,,	,, ,, ,, 30 min.	0/10
4	1 : 2 ,, ,, ,, 37° 1 hr.	h h h h h h h 1 2 2	0/10
5	1 : 2 ,, ,, ,, 37° 4 ,,	1 2 2 2 4	5/10

Undiluted toxin and ganglioside 1 mg./ml. mixed in equal volumes and sample withdrawn and injected at times shown for Groups 1, 2, 3, 4 and 5 (0·5 ml. i.v.). " h " indicates death within 4 hr. of challenge.

Action of ganglioside on tetanus toxin

Before using boiled ganglioside only limited success was obtained in attempts to protect mice against the lethal effect of tetanus toxin. Tetanus toxin is unstable in saline. It is stabilized and its toxicity enhanced by diluting in 0·5 per cent peptone or in 0·1 per cent gelatin saline. Both peptone and gelatin adversely affect the action of ganglioside on toxins. For instance in one experiment 0·025 mg. of ganglioside diluted in 0·5 per cent peptone was held at 37° for 35 min. with one LD_{100} of staphylococcal toxin. It failed to protect, all 10 mice dying within 48 hr. The same dose of ganglioside in saline protected 5 out of 10 mice. Incubated with toxin for 5 hr. at 37° ganglioside either in peptone or in saline protected all 10 mice. Other experiments showed that unlike its effect on tetanus toxin, peptone did not enhance the effect of staphylococcal toxin by the i.v. route in mice.

Table V shows the results of a representative experiment in which the effect of unheated ganglioside (Group 2) and of boiled ganglioside (Group 3) were compared against more than $10LD_{100}$ of tetanus toxin in mice. Ganglioside, both unheated and boiled, in the doses shown in the second column of the table was

held at 37° with tetanus toxin for 3½ hr. before injecting s.c. into mice. It will be seen that under the conditions of this experiment unheated ganglioside gave no protection whatever. On the other hand 0·1 mg. of boiled ganglioside delayed death to a significant degree—death in 4 days as against death in 1 day for mice given the same dose of toxin alone. The mice given toxin and 0·33 mg. of ganglioside survived. They showed some spasm but had almost recovered 14 days after challenge. The mice on 1 mg. of ganglioside remained well throughout the test.

TABLE V.—*Comparisons of Effect of Unheated and Boiled Ganglioside Against Action of Tetanus Toxin in Mice*

Group	Ganglioside (mg.)	Strength of toxin	Volume of toxin (ml.)	Day of death	Survivors 14 days
1	*Nil* (controls)	$1:2\times10^7$	0·1	2 3 3 3	0/4
	,, ,,	$1:2\times10^7$	0·33	2 2 2 2	0/4
	,, ,,	$1:2\times10^7$	1·0	1 1 1 1	0/4
2	0·1 unheated	$1:2\times10^7$	1·0	1 1 1 1	0/4
	0·33 ,,	$1:2\times10^7$	1·0	1 1 1 2	0/4
	1·0 ,,	$1:2\times10^7$	1·0	1 1 2 2	0/4
3	0·1 boiled	$1:2\times10^7$	1·0	4 4 4 4	0/4
	0·33 ,,	$1:2\times10^7$	1·0	—	4/4
	1·0 ,,	$1:2\times10^7$	1·0	—	4/4

Toxin alone and toxin + ganglioside were incubated at 37° for 3½ hr. before s.c. injection into mice. Experiment terminated 14 days later. Dilutions of toxin and ganglioside (below 10 mg./ml.) made in 0·1 per cent gelatin saline.

Action of ganglioside on diphtheria toxin

Three separate protection tests against diphtheria toxin were carried out on guinea-pigs before the advantage of boiled as compared with unboiled ganglioside was observed. All 3 experiments gave consistent results. The protocol of one of them is shown in Table VI. It will be seen that the challenge dose of toxin for animals of Groups 1, 2, 3 and 4 represented approximately $2{\cdot}5LD_{50}$ (Groups 4, 5 and 6). Against this challenge 5 mg. of unheated ganglioside incubated with the toxin at 37° for 4 hr. gave full protection, 2·5 mg. partial protection and 1·25 mg. prolonged the life of the guinea-pigs.

TABLE VI.—*Protective Effect of Unheated Ganglioside Against Action of Diphtheria Toxin in Guinea-pigs*

Group	Dose of toxin	Dose of ganglioside added (mg.)	Day of death	Survivors 14 days
1	L+/10	5	—	4/4
2	,,	2·5	7	1/2
3	,,	1·25	4 5	0/2
4	,,	*Nil*	2 2 2 2	0/4
5	L+/15	,,	2 2	0/2
6	L+/22·5	,,	3	1/2

Diphtheria toxin + ganglioside (Groups 1, 2 and 3) and diphtheria toxin in saline (Groups 4, 5 and 6) incubated at 37° for 4 hr. before s.c. injection into guinea pigs weighing > 245 and < 255 g. Experiment terminated after 14 days.

The survivors of another experiment, together with suitable controls, were challenged with the same dose of toxin ($2{\cdot}5LD_{50}$) 14 days after the original challenge. They died sooner than the control animals.

Alpha haemolytic titres of staphylococcal toxin-ganglioside mixtures

The alpha haemolytic titre of toxin-ganglioside mixtures prior to their injection into mice was examined. In all samples tested where the toxin-ganglioside mixtures were found to be less toxic than the original toxin, the haemolytic titre of the mixtures was found to be reduced. Strictly quantitative tests have not yet been done.

No other *in vitro* tests have so far been carried out on mixtures of any of the 3 toxins (staphylococcal, tetanus and diphtheria) with ganglioside.

DISCUSSION

As already indicated, this study was initiated because it seemed possible that a connection existed between the richness of brain tissue in phosphatides and its relative resistance to infection by the staphylococcus. Bogoch's (1957) studies prompted us to investigate the protective effect of the mucolipid, ganglioside, rather than crude brain preparations against the lethal action of staphylococcal toxin.

van Heyningen (1959*c*) regards ganglioside or possibly a contaminant, as the receptor for tetanus toxin. Further, he states that when the toxin and ganglioside are allowed to interact the toxin is inactivated. This is in accord with our own findings but we have shown that ganglioside inactivates staphylococcal and diphtheria toxins at least as effectively as tetanus toxin which appears to be contrary to van Heyningen's conception.

No significant difference was detected in the tests we have described between ganglioside preparations which varied in composition within the limits quoted. The ultra centrifuge run indicated inhomogeneity so that a common contaminant is not excluded. Some of the chemical changes that occur on heating have been studied. Sialic acid may be released on heating aqueous solutions of gangliosides (Bogoch, 1958) with a consequent fall in pH. The percentage released depends on the concentration and pH of the solution, the temperature and time of heating. The relationship between the extent of this release and the protective activity of heated preparations is still being examined.

Our studies with experimental animals have, so far, been concerned mainly with the inactivation of staphylococcal toxin. Mice have been protected against a dose of this toxin that killed all untreated animals within a few seconds, but we have not attempted to protect them against the massive doses of tetanus toxin used by van Heyningen (1959*a*) in his specific receptor studies.

It has already been suggested (Doery and North, 1960) that phosphatidase A plays a part in a natural defence mechanism of animals against the action of certain bacterial exotoxins. As a result of the action of the enzyme on phosphatide, lysophosphatide is released. Lysolecithin when injected alone protects experimental animals against subsequent challenge with staphylococcal toxin (North and Doery, 1960). Lysolecithin has an instantaneous solubilizing effect on brain homogenates (Neumann, 1957) and the significance of this as it affects the protective

mechanism is, at present, being examined. It is tempting to speculate on the possibility, and there is some evidence to support it, that lysolecithin releases sialic acid containing compounds from brain homogenates among which we assume gangliosides predominate.

One cannot visualize inactivation of toxin in the blood stream since ganglioside appears to be so readily adsorbed by serum albumin. On the other hand ganglioside inactivates toxin effectively in the subcutaneous tissues (see Table II). It may be that it is even more effective within the nerve cells themselves where gangliosides are situated (Svennerholm, 1957*a*).

Further studies on the mechanism of toxin inactivation by ganglioside and its relation to a natural defence mechanism are in progress.

SUMMARY

Ganglioside incubated at 37° with staphylococcal, tetanus or diphtheria toxin inactivates the toxin as shown by tests in experimental animals. Heating ganglioside stabilizes it and enhances its activity. Ganglioside fails to inactivate toxin in the presence of serum albumin. The significance of these findings is discussed.

We are indebted to Dr. L. C. Rowan for many helpful suggestions, to Mr. B. Graham for preparing the N-acetylneuraminic acid and to Mr. G. Wilson for the ultracentrifuge analysis. Miss Edith Russell, Miss Frances Bates and Miss S. Marklein gave valuable technical assistance.

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ANTIBACTERIAL IMMUNITY TO *STAPHYLOCOCCUS PYOGENES* IN RABBITS

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One problem encountered in attempting to study antibacterial as opposed to antitoxic immunity to staphylococcal infection experimentally is that both animals commonly used, the rabbit and to a lesser extent the mouse, are very susceptible to the lethal effects of α-toxin, particularly under the highly artificial conditions of intravenous challenge. Furthermore, nearly all strains having a relatively high virulence for these animals are strongly α-toxigenic. If these are used for challenge therefore, even though the organisms are washed as free as possible from toxin beforehand, the immediate production of fresh toxin *in vivo* in large amounts is liable to overwhelm antibacterial defence mechanisms. This factor may well have been responsible for failure to demonstrate antibacterial immunity in the past in some instances.

Forssman (1935, 1936*a* and *b*, 1937, 1938) has been one of the main protagonists of the view that specific immunity to staphylococcal infection does not depend primarily on the presence of antitoxic antibodies in the serum. Indeed he has insisted that antitoxin is of little or no value in conferring protection, particularly in so far as man is concerned.

He claimed to have induced specific immunity in rabbits, in the absence of the production of antitoxin, by injecting formolized vaccines intravenously and subcutaneously and maintained that the protective mechanisms developed, presumably antibacterial, were an essential factor in immunity to staphylococcal infection. Immunity was assessed on the basis of survival following intravenous challenge with a dose invariably lethal for controls, and on the clearance of organisms from the internal organs, in particular the kidneys. He maintained that immunity could be passively transferred, but found that immune serum rapidly lost its potency, in a matter of a few days, and he was unable to identify an antibody responsible for protection.

He laid considerable stress on the time taken for immunity to develop following the cessation of immunization, and found that it was considerably delayed compared with the normal course of antibody production. He gave no details as to the size of challenge dose required to kill, or as to the toxigenicity of the strains used, but it seems very probable that the results he described were obtained with a weakly α-toxigenic strain. He did however make the significant observation that the same degree of immunity was not demonstrated after injections of formolized emulsions of all strains tested. He ascribed these variations in results to differences in the vaccine, but since he appears to have used the homologous strain for challenge in each case, they may equally have been due to differences in the toxigenicity of the challenge strain.

Other workers have however completely failed to reproduce his results. Kitching and Farrell (1936) compared the immunizing activity of formolized vaccines and toxoid in rabbits and mice. Like other workers they found that toxoid conferred some degree of protection, but the formolized vaccine was without effect. It is significant however that they used the highly α-toxigenic strain Wood 46 for production of vaccine and toxoid, and also for challenge.

Smith (1937) studied the immunity induced in rabbits by washed formolized vaccine and different types of toxoid in relation to the anti-α-lysin produced, and found little or no evidence of immunity in the absence of antihaemolysin. Here again the Wood 46 strain was used for challenge.

Downie (1937) compared the immunizing activity of washed heat killed vaccines and toxoid in rabbits, using as a criterion of immunity a reduction in the reaction following inoculation of living staphylococci into the skin. He found that immunization with toxoid resulted in a marked reduction in the severity of the lesion, but that immunization with vaccine was without effect. He emphasized though, that these results might well only apply to challenge with a highly α-toxigenic strain such as he used, and that with strains producing little α-haemolysin and leucocidin, the factors determining pathogenicity and the mechanisms of immunity might be quite different.

Cowan (1939) also realized the importance that the toxigenicity of the infecting strain might have in influencing the result, and in his experiments used a weakly α-toxigenic strain, (S11), for vaccine preparation and challenge. The challenge dose required to kill consistently, was however considerably larger than that necessary with highly toxigenic strains. Using the intravenous method of administration throughout, he was able to induce immunity with 4 different types of heat-killed or formolized vaccines. He obtained similar results however with a vaccine prepared from an antigenically unrelated organism, namely *Pasteurella pseudotuberculosis*, and it seemed therefore that non-specific factors were involved. This may perhaps have been due to the fact that he used the same route for administering the vaccine as for challenge.

In the experiments described in the present paper, the problem has been investigated further using a weakly α-toxigenic strain for vaccine production and challenge. The vaccines consisted of washed killed whole cells or of fractions obtained from the washed cells by ultrasonic disintegration or by shaking and in most experiments were administered by the subcutaneous route.

EXPERIMENTAL METHODS

The selection of a suitable strain.—On testing strain S11, used by Cowan, it was found to have largely lost its virulence, and even the relatively large doses he used failed to kill. This was also the experience of Brodie, Guthrie and Sommerville (1958) who have reported favourable results using it as a living vaccine. A large number of strains from a collection referred to by Selbie and Simon (1952), were then tested, and one, namely strain 13 (phage type I–III) was found to meet the requirements of weak α toxin production, combined with a relatively high virulence for rabbits. It was therefore used throughout for vaccine production and challenge. A proteolytic strain, 17 (phage type I–III) was also used in one experiment (Table II) for the production of a cell wall fraction.

Selbie and Simon, using whole cultures grown in tryptic digest broth for 48 hr. in 30 per cent CO_2, recorded a titre of 1/5 for strain 13 when tested against rabbit cells, the titre being taken as the highest dilution showing at least 50 per cent haemolysis after 1 hr. at 37°. In the present work, in which filtrates of cultures grown for 3 days on semi-solid nutrient agar (0·3 per cent) in 20 per cent CO_2 were used, the titres varied from 1/8 to 1/32 with different batches of

filtrate. Titres of 1/512 to 1/2048 were obtained with cultures filtrates of 2 strongly α toxigenic strains, 7228 and 144, tested at the same time. The haemolytic activity of the filtrates was completely neutralized by antitoxin. Culture filtrates of these 3 strains were also tested for mouse toxicity immediately after preparation by injecting 0·5ml. of neat or diluted filtrate intraperitoneally into mice. The filtrate of strain 13 consistently failed to kill even when undiluted. Those of strains 7228 and 144 killed 10 out of 10 mice in dilutions of 1/8 to 1/16.

Strain 13 produced a trace of β-lysin and also some δ-lysin, though rather less than the average amount produced by δ toxigenic strains, when assessed by the method described by Williams and Harper (1947). It also produced very little PV leucocidin (about 10 minimal leucocidal doses/ml.)*in vitro* under optimal conditions (Gladstone and van Heyningen, 1957) when tested by Dr. G. P. Gladstone.

It however produced an unusually large amount of coagulase. Filtrates prepared from 48 hr. digest broth cultures contained about 400 minimal clotting doses (MCD)/ml. for rabbit plasma when tested by the method of Lominski and Roberts (1946). Culture filtrates of a number of other coagulase positive strains tested at the same time in nearly every case contained much less (usually between 20–100 MCD/ml.). One exception was that of the " freak " Newman strain used by Duthie and Lorenz (1952) which contained about 2,000 MCD/ml.

The virulence of strain 13 for rabbits was tested as follows. A 24 hr. digest broth culture was centrifuged, resuspended in the same volume of saline, and varying dilutions of this suspension in saline were injected intravenously in 1 ml. quantities. Almost invariably, 0·1 ml. of original culture, *i.e.* approximately 3×10^7 viable units killed, 0·04 ml. killed 3 out of 4, 0·02 ml. killed 2 out of 3, and 0·004 ml. killed 1 out of 2 rabbits. These results compared favourably with those obtained with the 2 more toxigenic strains 7228 and 144 tested on a smaller scale. These failed to kill regularly in a dose of 0·1 ml.

Preparation of thick cell suspensions for vaccine production.—Cultures of strain 13 were grown for 24 hr. at 37° in 400 ml. Difco tryptose broth contained in 1 l. flasks, or for 4 hr. at 37° in 1 l. quantities in 5 l. flasks. The growth was centrifuged in the cold, washed once in saline and resuspended in saline in a concentration of 20–30 mg./ml.

Preparation of " whole cell " vaccines.—The heat killed vaccine was prepared by heating the thick suspension for 1½ hr. at 60° and was stored without a preservative. An alcohol killed and preserved vaccine was also used. This had given particularly promising results in an earlier series of experiments in which different types of vaccine were tested in mice using the mucin technique of challenge. It was prepared by diluting the thick suspension 1/10 in saline and adding 3 volumes of absolute alcohol. After standing for 2 hr. at room temperature the cells were centrifuged, resuspended and stored as a thick suspension in absolute alcohol.

Preparation of " supernatant " and crude cell wall fractions.—A volume of 50 ml. of thick suspension was submitted to ultrasonic vibration for varying periods in a Mullard ultrasonic magnetostricton oscillator (2 kw., 4 amps. high frequency output).

The suspension was then centrifuged in the cold at 3,000 rpm for 1 hr. to remove unruptured or partially ruptured cells, and the supernatant was recentrifuged in a Spinco ø† 18,000 rpm for 1 hr. In most experiments the clear supernatant was then treated with 4 or 9 volumes of alcohol. The precipitate which formed was resuspended and stored in absolute alcohol as in the preparation of the alcoholized whole cell vaccine. This fraction is referred to in the text by the letter S, *e.g.* 4-S-30, the figure preceding indicating the period of growth of the culture in hr. and that following, the period of treatment in min. In the experiment recorded in Table IV only a part of the batch of supernatant was treated with alcohol in this way. The remainder was formolized to 0·2 per cent by the addition of 10 per cent formolized saline, and incubated for 5 days at 37°.

The deposit of cell wall material, obtained by high speed centrifuging, was washed once in saline and resuspended in further saline. The suspension had the opalescent appearance characteristic of cell wall preparations. A small sample was removed and diluted in water for electron microscopy. Four volumes of alcohol were added to the remainder. This was recentrifuged and the deposit resuspended in absolute alcohol.

Preparation of saline extract.—A volume of 50 ml. of thick suspension was shaken with glass beads (5 mm. diam.) for 30 min. in the cold, centrifuged and treated with alcohol as in the preparation of the " supernatant "' fraction above.

The standardization of vaccines.—This was done on a dry weight basis. One ml. of the suspension of whole cells, supernatant or cell wall precipitate was delivered into a weighed

centrifuge tube, 4 ml. of absolute alcohol was added and the suspension centrifuged. The deposit was resuspended in 5 ml. of acetone, recentrifuged and dried *in vacuo* over P_2O_5. The weight of cells or fraction per ml. of suspension was calculated and the vaccine was diluted with absolute alcohol or with saline in the case of the heat killed vaccine and formolized " supernatant " fraction to a concentration of 4 mg./ml. All vaccines were stored at 0°.

Immunization procedure.—Laboratory-bred animals weighing between 2–3 kg. were used. Their sera were tested for the presence of anti-α lysin before immunization was commenced, and only those giving a negative result, *i.e.* less than 0·1 *i.u.* were included in the tests.

The vaccines were diluted in saline to a concentration of 0·5 mg./ml. Four doses of 0·5 mg. were given i.v. at 6-day intervals (Table I), and 5 doses were given s.c. into a shaven area over the flank at 5-day intervals (Table II) and at 6-day intervals (Table III and IV). Samples of blood for anti-α toxin estimations were taken 1–3 weeks, and the animals were infected 3–6 weeks after the last immunizing dose.

Method of challenge.—A 24-hr. culture of strain 13 in digest broth was centrifuged and the deposit resuspended in the same volume of saline. One ml. of a 1/12·5 dilution in saline was given i.v. The infecting dose was 2–2·5 $\times$ 10^7 units as assessed by surface viable counts.

Observation of rabbits.—The animals were kept under observation for 2–2½ months. Survivors were killed and examined for persistent infection, particularly of kidneys, bones and joints.

Examination of sera for anti-α toxin.—This was done by the haemolytic method, using rabbits' red cells and a standard anti-α haemolysin.

RESULTS

In the first experiment batches of rabbits were immunized with washed alcoholized and heat-killed vaccines using the intravenous route. It will be seen that both vaccines induced a partial degree of immunity under these conditions, and a further similar experiment confirmed these results.

TABLE I.—*Results of Immunizing Intravenously with Heat-killed and Alcoholized Vaccines*

Vaccine	Number of rabbits per batch	Survivors	Day of death
Heat-killed	4	2	2, 2
Alcoholized	5	3	2, 2
Control (no vaccine given)	5	0	2, 1, 1, 1, 1

An attempt was then made to fractionate the cells by ultrasonic disintegration (Fig.) in order if possible to identify the protective factor with a particular cell component as for example the cell wall, and also perhaps to obtain a more potent immunizing preparation.

In the first fractionation the cells from a 24 hr. culture were treated for 3 hr. This period was found to be necessary since as Saltoun (1956) has observed, staphylococci are normally relatively resistant to ultrasonic disruption. After such prolonged treatment however, the opacity of the suspension was reduced to 10–20 per cent of the original. Electron photomicrographs of the material deposited after the second centrifuging carried out at 18,000 rpm showed mainly fragments of cell walls with a few intact cell walls and occasional partially disrupted cells. This crude cell wall fraction was washed once in saline, precipitated in 75 per cent alcohol and preserved in absolute alcohol. The clear supernatant was precipitated with 9 volumes of alcohol and the precipitate preserved in absolute alcohol. A more satisfactory cell wall preparation was obtained using a strongly proteolytic strain (17). This was found to disintegrate more readily and the period of treat-

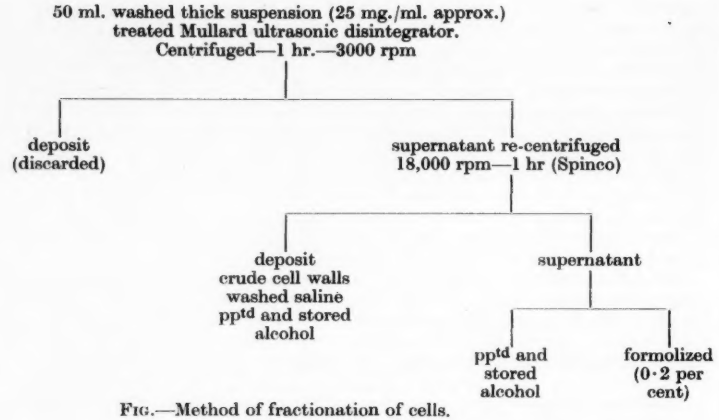

FIG.—Method of fractionation of cells.

ment could therefore be considerably reduced. The 2 cell wall fractions were tested along with the " supernatant " fraction and an alcoholized whole cell vaccine of strain 13. (Table II.) These vaccines were given s.c. in view of the possibility, suggested by Cowan's experiments, that administration by the intravenous route used in the previous tests might produce non-specific effects.

TABLE II.—*Results of Immunizing Subcutaneously with Whole Cell Vaccine, Crude Cell Wall Fractions, and an Alcohol Precipitate of the " Supernatant " Fraction, Preserved in Alcohol*

Strain used for vaccine	Type of vaccine	Number of rabbits per batch	Survivors	Day of death
13	" Whole cell "	5	1	1, 1, 2, 3
13	Cell wall	4	0	2, 2, 2, 69
17	,, ,,	5	0	2, 2, 2, 2, 3
13	" Supernatant "	5	3	1, 3
Control (no vaccine given)		5	0	1, 2, 2, 2, 4

It will be seen that in this experiment, contrary to the findings in Table I the whole cell vaccine conferred little or no immunity. The cell wall fractions also were completely inactive. The " supernatant " fraction on the other hand produced a definite increase in resistance, 3 out of 5 animals surviving, and it was therefore investigated further. It seemed possible that the protective factor in this fraction might have come from the slime layer or capsule, which Lyons (1937) and Price and Kneeland (1954, 1956) claimed to have demonstrated in coagulase positive staphylococci. If this were so, it would seem advisable to use young cells to prepare the fraction, since according to these workers capsular material has largely disappeared after 24 hr. growth. Furthermore rupture of the cells should be avoided as far as possible, so as to reduce the amount of inactive material in the fraction.

In the next experiment therefore, a batch of " supernatant " fraction was prepared from cells grown for 4 hr. as well as a further batch from a 24 hr. culture,

and the suspensions were treated for 20 min. only. In previous tests treatment for this short period had produced little or no reduction in opacity. It was found, however, that young cells are much more easily disintegrated than those from older cultures, as was shown by a marked reduction in the opacity of the young cell suspension and also by the respective yields of the 2 batches of fraction recorded in Table III, and it was evident therefore, that much less drastic treatment was required if disruption of the cells was to be reduced to a minimum. The results of immunization with the 2 batches are given in Table III.

TABLE III.—*Results of Immunizing Subcutaneously with an Alcohol Precipitate of the " Supernatant " Fraction from Cells Grown for 4 and 24 Hr*

Fraction	Yield of fraction (percentage of total wt. of cells)	Number of rabbits per batch	Survivors	Day of death
24-S-20	6	6	3	2, 31, 56
4-S-20	33	6	4	4, 5
Control (no vaccine given)		6	0	2, 2, 2, 4, 4, 5

It will be seen that the immunizing activity of the " supernatant " fraction was confirmed, though there was no appreciable difference in the potency of the 2 fractions.

In a further experiment an attempt was made to isolate the protective factor by shaking a saline suspension of washed young cells gently with coarse glass beads for a short period in the cold. This produced little or no disruption of the cells judged by opacity estimations before and after treatment. After high speed centrifuging, the supernatant was precipitated with 3 volumes of alcohol and the precipitate stored as usual in absolute alcohol. Another portion of the same batch of cells was given ultrasonic treatment as before, but for 5 min. only. In spite of this further reduction in the period of treatment considerable disruption of the cells resulted. The " supernatant " after high speed centrifuging was divided into 2 portions. One received the usual treatment with alcohol and the other was formolized to 0·2 per cent and incubated at 37° for 5 days as in the treatment used to convert toxin into toxoid. The 3 preparations were then tested as before (Table IV).

TABLE IV.—*Results of Immunizing Subcutaneously with Alcoholized and Formolized Preparations of the " Supernatant " Fraction (4-S-5) and also with a Fraction Isolated from the Cells by Shaking*

Fraction obtained by	Treatment of fraction	Yield of fraction (percentage of total wt. of cells)	Number of rabbits per batch	Survivors	Day of death
Ultrasonic treatment (4-S-5)	Alcohol	21	6	2	3, 3, 5, 6
	Formalin	—	5	5	—
Shaking	Alcohol	3	6	5	3
Control (no vaccine)		—	5	1	2, 4, 5, 20

The fraction removed from the cells by shaking was very active, a result consistent with the view that the protective factor is situated at the cell surface. The formolized fraction also induced a high degree of immunity. This finding is of considerable significance since as the protective factor is probably present in

some culture filtrates used to prepare toxoid it may be partly responsible for the protection afforded by toxoid in some instances.

In all the experiments the sera of immunized animals were tested for anti-α lysin and were consistently found to be negative at a level of 0·1 i.u. and above. This was not unexpected for the following reasons. Not only were the vaccines prepared from a weakly α-lytic strain, but they were grown under conditions unfavourable for the production of α-lysin and the cells were washed before ultrasonic treatment. Also the fresh untreated fraction, showed no haemolytic activity when 0·5 mg. was mixed with a suspension of rabbit red cells in saline. This fraction also failed to produce a significant reaction when tested for dermonecrotic activity by injecting the same amount into the skin of rabbits. There is therefore no reason for believing that the immunity demonstrated in this work was due to antitoxin. Tests of the antisera for anti-leucocidin kindly carried out by Dr. G. P. Gladstone likewise proved negative.

The level of immunity in surviving animals appeared to be relatively high. At the end of the observation period they appeared healthy and had regained nearly all if not all of the weight they had lost. At post mortem only a minority showed evidence of persistent infection in the form of abcesses in the kidneys.

DISCUSSION

These results support Forssman's contention that under certain conditions staphylococcal vaccines are capable of inducing specific immunity in rabbits in the absence of the production of circulating antitoxin. They have failed however to confirm his findings as to the time taken for immunity to develop following the cessation of immunization. He claimed that immunity was not consistently evident until the 57–60th day, reaching its maximum on the 75–94th day after the last immunizing dose. He noticed in one series of tests a transient wave of immunity about the 17th day which rapidly waned, but this was not reproduced in a subsequent series. In these experiments immunity was demonstrated 20–40 days after the last immunizing dose, *i.e.* at a time when according to Forssman the animals showed little or no increased resistance. The development of immunity in the present work is therefore more related to the course of antibody production than in Forssman's experiments.

The nature of the protective factor and of the mechanisms involved in this type of immunity have however yet to be determined. The fact that the protective factor may be removed from the cells by mild treatment such as gentle shaking suggests that it may be located at the cell surface, possibly in the form of slime layer or capsular material.

The evidence as to capsulation in staphylococci is conflicting. Lyons (1937) claimed to have demonstrated capsules in young cultures of *Staph. pyogenes* and maintained that they were responsible for the invasive properties of the organism. Spink (1939), Klieneberger-Nobel (1948) and other workers have however failed to confirm his findings and it is very doubtful whether in fact capsular material can be demonstrated in this organism by staining methods such as he used. On the other hand Price and Kneeland (1954, 1956) were able to demonstrate capsules in young cultures of normal coagulase-positive strains by means of a capsule swelling reaction using an antiserum prepared against a mucoid strain, and in experiments forming part of the present investigation to be described in a later paper, these

findings have been confirmed. A study of capsule swelling antibody in relation to immunity might therefore be a fruitful line of investigation.

It may also be significant that strain 13 produces an exceptionally large amount of free coagulase. According to Boake (1956) antibody to free coagulase is protective under certain conditions, and Haughton (unpublished) has produced evidence supporting this finding. Present indications from a limited number of tests, however, are that neither anti-free coagulase nor anti-bound coagulase is present in appreciable amounts in the immune sera. It is possible that a protective antibody may be involved which interferes with the activity of coagulase in an indirect way, perhaps by inhibiting its formation from a precursor.

Further work is in progress to determine the efficacy of this method of immunization, on the one hand when the vaccine is prepared from strains differing in characteristics that may be of importance for protection, such as coagulase activity, and on the other when strains of greater toxigenicity and heterologous serotype are used for challenge.

SUMMARY

Staphylococcal vaccines given subcutaneously protected rabbits against challenge with a weakly α-toxigenic strain. The vaccines consisted of a soluble fraction obtained from washed cells by ultrasonic disruption and also in one experiment by shaking. The sera of immunized animals contained no appreciable amounts of anti-α lysin.

I am indebted to the late Professor F. R. Selbie for supplying many of the strains used, to Dr. G. P. Gladstone for leucocidin and anti-leucocidin investigations, to the Wright-Fleming Institute for the use of an ultrasonic disintegrator and for electron microphotographs, and to Mr. J. M. Goodwin and Mrs. J. Bertie for technical assistance.

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IRRADIATION AS A CAUSE OF "MASCULINIZATION" IN SUBMAXILLARY GLANDS

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BASED on the histological observations of Ranvier (1886) and Tupa (1926), Lacassagne (1940*a*, *b* and *c*) pointed out that the histological structure of submaxillary glands in normal rats and mice are different in males and females. He described this sexual dimorphism and showed its interrelationship with sex hormones.

Although English (1955) and Cherry and Glucksmann (1959) published some interesting observations on the changes occurring in the submaxillary glands of irradiated rats, other authors including Casarett (1952), Shafer (1952), and Friedman and Warren (1942), failed to observe any such significant changes.

Berdjis and his co-workers (Berdjis, Kallman, Kohn and DeOme, 1956; Kohn, Kallman, Berdjis and DeOme, 1957), however, in a systematic study of the organs of normal and irradiated mice, reported a peculiar change in kidneys and submaxillary glands of irradiated female mice which they called masculinization.

In a similar study of the organs of irradiated rats, it was found that the same histopathological changes were present in the submaxillary glands of females.

The purpose of this paper is to describe this masculinization in the submaxillary glands of irradiated rats.

MATERIALS AND METHODS

Two groups of adult healthy Sprague-Dawley rats, 25 males and 25 females, received 500 r total body X-irradiation and 2 untreated groups, 20 males and 20 females, served as controls. The rats were roughly 6 months of age at the time of exposure. Following irradiation, the animals were kept under observation and were maintained on a standard laboratory diet for approximately 1 yr. At about 18 months of age they were killed, their principal organs, including submaxillary glands, were examined grossly and microscopically. The tissues were fixed in 10 per cent formalin, embedded in paraffin and stained routinely with haematoxylin and eosin, unless special mention is made.

The principal radiation factors were: 250 kV X-ray machine, added filtration 0·5 mm. Cu and 1 mm. Al; 15 MA; TSD 100–110 cm.; rate 60–70 r/mm.

RESULTS

Control Rats

Normal structure of submaxillary glands in the rat and mouse

The submaxillary gland, a tubulo-alveolar organ, is composed of 2 main parts: acini and tubules. The acini are identical in male and female (Fig. 1 and 2); they are composed of relatively large pyramidal cells, generally with basophilic

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alveolar cytoplasm, dark, hyperchromatic, roughly triangular nuclei, basilar in position, and a virtual lumen.

There are 3 kinds of tubules in rat and mouse submaxillary glands :

Interlobular striated tubules, called also *intercalary tubules* because they join the acini, are very short and narrow. They are lined by a row of columnar epithelial cells characterized by basal striation. There is no difference in structure between male and female.

Secretory or *terminal tubules* which form the active glandular portion of submaxillary glands, differ in male and female animals. In males (Fig. 1) these tubules are large, numerous and lined by turgescent epithelial cells, generally tall and columnar or somewhat pyramidal or prismatic (tropochromic cells of certain authors) resembling mucous cells, although mucicarmine stain is negative. The principal characteristic of these cells which differentiates the sexes is that they contain a large number of acidophilic granules. In females (Fig. 2) secretory tubules are much smaller ; lining epithelium cells are cuboidal or slightly elongated and generally devoid of acidophilic granules.

The excretory tubule or duct.—The main duct or duct orifice is lined by stratified, squamous epithelium ; the other ducts or excretory tubules, which are identical in males and females, are lined by pseudostratified and more or less, columnar epithelium.

In summary, in a normal male submaxillary gland the tubules with acidophilic granules predominate and in the female, the acini.

Irradiated Rats

Radiation affects the acini and the secretory tubules leaving intact the intercalary and excretory tubules. Therefore, the following observations are related to the changes occurring in the 2 main components.

Female irradiated animals (*Fig.* 3)

Acini.—The principal acinar characteristics, namely size, colour and amount, are changed after irradiation. Many acini are occluded or atrophic exhibiting interstitial fibrosis and/or stromal round cell infiltration. A great variation is observed in the size, shape and tinctorial affinities of both nuclei and cellular components of the acini. Large hyperchromatic wedge-shaped, irregularly triangular, prismatic or stellate nuclei are frequently seen. Occasional giant cells are also present. This nuclear pleomorphism is accompanied by cytoplasmic changes which vary from alveolar structure to finely granular or vacuolated protoplasmic material. Although generally basophilic, a faintly eosinophilic background can be observed in most acini. This eosinophilic appearance is probably due to masculinization of the gland.

Tubules.—The intercalary and excretory tubules being refractory to the effects of irradiation, the glandular or secretory tubules are the only tubular component which is highly affected by X-ray. The changes occurring in these tubules represent the most interesting and striking finding of this study. Under normal conditions only a small portion of the female submaxillary gland is occupied by the tubules which are generally narrow and devoid of eosinophilic granules (Fig. 2, Tables I and II). Irradiation causes hypertrophy of the epithelial cells and

TABLE I.—*Tubule Diameter and Epithelial Cell Height in Submaxillary Glands of Rats : " Masculinization Index "*

Dimensions*	Control rats		Irradiated rats	
	Male	Female	Male	Female
Tubule diameter (μ)	58 (37–79)†	39 (32–46)	52 (35–69)	48 (36–60)
Cell height (μ)	28 (22–34)	19 (16–22)	25 (19–31)	23 (18–28)
Ratio— Diameter : height	2·1	2·1	2·1	2·1

* Diameter and cell height were measured in at least 5 microscopic fields for each tissue section.
† Range.

TABLE II.—*Percentage of Tubules and Acini in Submaxillary Glands of Normal and Irradiated Rats*

Gland component*	Average per cent of gland			
	Male rates		Female rats	
	Controls	Irradiated	Controls	Irradiated
Acini (per cent)	34 (32–36)†	39 (36–42)	71 (67–75)	55 (46–64)
Tubules (per cent)	66 (63–69)	61 (58–64)	29 (27–31)	45 (35–55)
Ratio— Acini : tubules	0·5	0·6	2·7	1·2

* Chalkley (1953) method is used for estimations of the per cent acini and tubules (at least 5 microscopic fields were evaluated for each section).
† Range.

increases the tubular diameter so that they become turgescent and rich in eosinophilic granules similar to the male tubules (compare Fig. 3 with Fig. 1).

Male irradiated animals

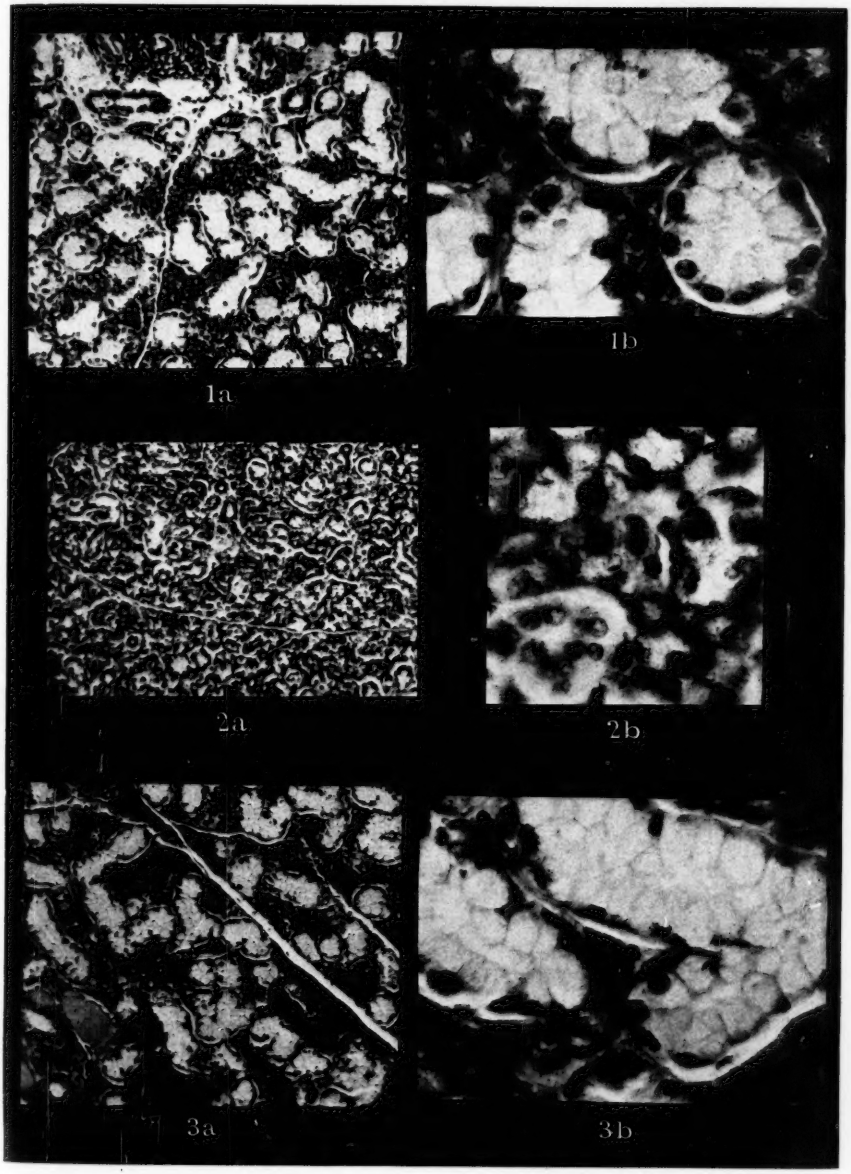
Acini.—Although partially atrophic and occluded, there are fewer variations in size, shape and staining affinities here than in the female acini. The cytoplasm

EXPLANATION OF PLATE

FIG. 1.—Normal male submaxillary gland in a rat (18 months old). Note predominance of tubules with large epithelial cells, rich in acidophilic granules. H. and E. *a :* ×60, *b :* ×475.

FIG. 2.—Normal submaxillary gland in a female rat. Note predominance of the acini and narrow tubules with small epithelial cells, devoid of acidophilic granules. H. and E. *a :* ×60, *b :* ×475.

FIG. 3.—Masculinization of a submaxillary gland in an irradiated rat 1 yr. after 500 r total body X-irradiation. Note the turgescence of the epithelial cells and increased number of the tubules. H. and E. *a :* ×60, *b :* ×475.


1a
1b
2a
2b
3a
3b

is relatively clear. It remains mostly alveolar and partly granular with more prominent acidophilic background than in the female. The tinctorial affinities are generally faintly basophilic.

Tubules.—Although mildly decreased in size and amount, the tubules are not significantly affected by radiation (Tables I and II).

Under normal conditions roughly about 2/3 of the male submaxillary gland is occupied by tubules. In the female the situation is reversed and acini occupy over 2/3 of the submaxillary gland (Fig. 1, 2 and 3 ; Tables I and II).

The situation is almost unchanged in irradiated male animals, where 60 per cent of the submaxillary gland consists of tubules. The situation is entirely different in irradiated female rats. The latter exhibit large glands almost the same size as non-irradiated controls, but it is obvious that the composition is reversed : 40–50 per cent of the gland is composed of tubules with large epithelial cells rich in acidophilic granules similar to those observed in males.

These changes, characteristic of normal male submaxillary glands (Fig. 1), which are present in female irradiated rats, are referred to as masculinization of these glands.

According to Tables I and II, irradiation masculinized the female submaxillary glands so that the tubules were enlarged and the epithelial cells hypertrophied. The latter contained large amounts of acidophilic granules, unusual for the female submaxillary gland. This spectacular transformation in irradiated female submaxillary glands is also accompanied by increased height of the epithelial cells.

DISCUSSION

According to our own observations and those of English (1955), and Cherry and Glucksmann (1959) we are far from the concept that the salivary glands are apparently radio-resistant and that these glands show less morphological than physiological response to radiation (Friedman, 1942 ; Warren, 1944 ; and Shafer, 1952).

Although Desjardins (1931) and Von Salis (1924), found evidence of changes in the acini and small ducts, Casarett (1952) found no definite consistent changes in the submaxillary glands of the rats treated with polonium.

The first valuable observations were published by English (1955). This author found variation in size and staining of the acinar cells with extensive nuclear pleomorphism, but did not mention any change in the tubules, described herein as masculinization. He stated instead that the tubules and the ducts showed nothing remarkable. Cherry and Glucksmann (1959) in a very interesting report on injury and repair following irradiation of salivary glands in male rats, stated that the acini are most affected.

Berdjis and his associates (Berdjis *et al.*, 1956 ; Kohn *et al.*, 1957) reported a series of changes in submaxillary glands of irradiated mice, pointing out also that these changes were not alike in males and females ; they called attention to masculinization.

The submaxillary gland of the irradiated rats underwent several major changes which are summarized here : (*a*) atrophy with occluded acini in both sexes, although much more prominent in the females ; (*b*) morphological changes in the tubules of the females and the height of the epithelial cells ; (*c*) qualitative changes, by stain difference and masculinization.

Most of the females developed radiation-induced hormone-secreting tumours such as granulosa-cell tumours, masculinized tumours (arrhenoblastoma), etc., in highly atrophic and non-functioning ovaries. These tumours seemed to be one of the factors which engendered masculinization in submaxillary glands and kidneys of irradiated rats. The mechanism of action appeared to be related to hormonal imbalance of the endocrine system and intimately related to the degree of the ovarian injury. Furthermore, it has been shown previously that sexual dimorphism, a physiological phenomenon in rats and mice, is under the control of sex hormones (androgens and oestrogens) (Lacassagne, 1940*a*, *b* and *c*; Crabtree, 1941).

From the earlier observations and the present study it is suggested that the degree of masculinization which represents the richness of the epithelial cells in acidophilic granules and the diameter of the tubules, could be used as an index of hormonal imbalance. This index may assist in evaluating the ovarian function in irradiated rats and mice.

SUMMARY

Radiation masculinizes the submaxillary glands of female irradiated rats as it does in mice.

Radiation affects both components of submaxillary glands, acini and tubules, with more severe changes in the female. These changes can be summarized as: (*a*) atrophy, with occluded acini in both sexes, (*b*) tubular hypertrophy in females and (*c*) masculinization with acidophilic granules in the female.

Most of these changes are under the influence of hormonal imbalance related to ovarian injury.

The degree of masculinization (richness of the epithelial cells in acidophilic granules, their height, and the tubular diameter) can be used as an index of hormonal imbalance in evaluating the ovarian function in rats and mice.

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RELATION OF RABBIT GUT REACTION TO ENTEROPATHOGENIC *ESCHERICHIA COLI*

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PREVIOUSLY we published an account of factors influencing the reaction of the ligated rabbit gut to injections of strains of *Escherichia coli* (Taylor, Maltby and Payne 1958), and showed that positive reactions were obtained only with living cultures. Bokai (1888) described a somewhat similar technique. Using dogs, he tested various organisms including *Esch. coli*, and from the results obtained suggested that skatol and other protein break-down products caused the reaction. He was able to show that skatol and lactic acid products, caused increased gut movements, and believed that these substances were also produced by the organisms tested. De and Chatterje (1953) were the first to describe the use of ligated rabbit gut and investigated strains of *Vibrio cholerae* by this method ; in later communications De (1959) and De, Ghose and Sen (1960) reported that from strains of *V. cholerae* producing positive reactions a sterile filtrate could be obtained which would produce a similar reaction. Jenkin and Rowley (1959, 1960) also reported results with *V. cholerae* ; in addition, they showed that dilatation of the rabbit gut was caused by lactic acid and further suggested that this may be the toxic product produced by the strains of *V. cholerae* and *Esch. coli*.

We have extended our original studies and have examined a large number of strains of *Esch. coli* of various serotypes isolated from human, animal and miscellaneous sources. The object of this part of our work was to discover (*a*) if positive and negative results in the rabbit gut test were in accord with the history of the patients or animals from which the strains had been isolated, (*b*) whether human enteropathogenic serotypes isolated from animal or water sources produced a reaction, and (*c*) whether a positive reaction was caused by *Esch. coli* isolated from infections in which diarrhoea was not a symptom.

The second part of our work was devoted to determining the number of organisms, colonial form, the effect of the method of keeping cultures, media, and immunity on the results obtained.

PART 1

Results of tests on strains of Esch. coli *of human origin*

The methods used for testing strains on ligated loops of rabbit gut, and for reporting histological results, were the same as those described previously (Taylor *et al.*, 1958).

Strains were isolated from the faeces of babies whose clinical history was well-known. It was found that some strains which had been tested in previous years and which had given a positive reaction, were negative when re-checked. For example, the strain of *Esch. coli* O.111 : K.58 (B.4) : non-motile (nm.), Table I, was culture D.433 which was tested by De, Bhattacharya and Sarkar (1956) and found to be positive. This strain was tested by

us in 1955, when we sometimes obtained a positive reaction, sometimes a negative. It was tested on 2 subsequent occasions in 1957, 10 yr. after its isolation from faeces, and gave negative results. The culture appeared to be smooth. Results on this and other strains are given in Table I.

TABLE I.—Esch. coli *from Babies; Effect on Ligated Rabbit Gut*

Esch. coli				Ligated gut		
Serotype	History	Number of strains	Dilatation	Exudate	Histology
O.4 nm.	Diarrhoea	1	−	−	O
H.5	,,	1	−	−	,,
O.20 H.34	,,	2	−	−	Mild
O.26 : K.60 : nm.	,,	1	+	+	Moderate
O.55 : K.59 : H.7	,,	4	+	+	Moderate or severe
H.6	,,	1	+	+	Moderate
O.78 : K.80 : nm.	,,	2	+	+	O
O.111 : K.58 : nm.	,,	1	+	+	Moderate
O.119 : K.69 : H.6	,,	1	+	+	,,
	,,	1	−	Trace pus	,,
O.128 : K.67 : nm.	,,	1	+	+	,,
H.2	,,	1	+	+	,,
H.8	,,	1	+	+	,,
Biotype I	,,	3	−	−	Mild–moderate
O.26 : K.60 : H.11	Symptomless*	1	+	+	Moderate
O.78 : K.80 : H.12	,,	1	−	−	Mild–moderate
O.111 : K.58 : H.2	,,	1	−	−	,, ,,
H.4	,,	1	−	−	,, ,,
O.128 : K.67 : nm.	,,	1	−	−	O
H.8	,,	1	−	−	Mild
H.8	,,*	1	+	+	Moderate
Biotype I	,,	3	−	−	Mild–moderate

* Patients were contacts of patients with diarrhoea who excreted same serotype.
O = not done. nm. = non-motile.

Two strains of *Esch. coli* O.4 were tested, this serotype is not accepted as an enteropathogenic type; both strains gave negative results.

It will be seen that 2 strains of *Esch. coli* O.20 were tested, which were isolated from 2 babies with mild diarrhoea in an outbreak affecting about 8 babies in a maternity unit. These gave negative reactions to the test, but they had been kept on Dorset's egg medium for 2 yr. prior to testing. *Esch. coli* O.20 is not generally accepted as a cause of diarrhoea.

The strain of *Esch. coli* O.26 : K.60 (B.6) : H.11 which gave a positive result was isolated from faeces which were investigated in a survey of healthy children. Nothing is known of the clinical history of this patient, except that he was symptomless at the time the specimen was taken.

A strain of *Esch. coli* O.78 : K.80 was tested, as it causes white scours in calves, but is rarely isolated from human material. The strains from cases of infantile diarrhoea were positive, whereas the strain from a healthy baby was negative.

Two strains of *Esch. coli* O.119 : K.69 (B.14) : H.6 were tested. These were isolated from babies in 2 unconnected outbreaks. One strain caused dilatation of the gut with production of exudate, the second failed to cause dilatation of the gut although pus was present on the mucosal surface; in both instances the histological picture was one of a moderate degree of inflammation.

Esch. coli O.128 : K.67 (B.12) : H.8 was isolated from a baby with diarrhoea, from a healthy baby who, having been in contact with a case, was admitted to a residential nursery, so starting a small outbreak, and from a healthy baby unconnected with cases. The results of

the gut test were in accordance with the history of the patients from whom the strains were isolated, the strains from the baby with diarrhoea and from a healthy contact who was the cause of the outbreak giving positive reactions whereas the strain from the healthy baby failed to cause a reaction.

A study of a residential nursery was in progress in which all strains of *Esch. coli* were typed according to their fermentation reactions to lactose, maltose, sucrose, salicin, dulcitol, inositol, sorbitol, raffinose and rhamnose. During this study, an outbreak of diarrhoea occurred which was believed to be caused by a virus, though this was unproven. It was thought that a biotype I which was found in the healthy and the affected children should be tested as it was possible that this type which was common in the nursery during the whole period of study of 3 yr. might have acquired enteropathogenic properties as the result of passage from baby to baby. Three strains of biotype I from healthy babies, and 3 strains from babies with diarrhoea, were tested. All gave a negative reaction.

The results given in Table I indicate that there is good correlation between the ability of a strain of *Esch. coli* to cause a positive gut reaction and its ability to cause diarrhoea in babies. The tests reported suggest that the ability to cause dilatation of the gut may be reduced and finally lost in strains kept in the laboratory. We suspect that, although some strains produce a positive test after keeping for a number of years, others may lose this property fairly quickly.

The possibility that a positive gut reaction might be caused by any pathogenic strain of *Esch. coli* and not confined to those strains causing diarrhoea, was considered, so 4 cultures isolated from human urinary infections were tested. All gave negative results.

TABLE II.—Esch. coli *from Farm Animals, Effect on Ligated Rabbit Gut*

Animal	Disease	Serotype	Ligated gut: Dilatation	Ligated gut: Exudate	Ligated gut: Histology
Calf	White scours	O.9 : K ? : H.19	—	—	Mild
,,	,, ,,	O.26 : K.60 : H.11	+	+	Mild–moderate
,,	,, ,,	O.78 : K.80 : nm.	+	+	Moderate
,,	,, ,,	O.137 : K.79 : H.41	+	+	Mild
,,	,, ,,	4 unclassified serotypes	—	—	Mild–moderate
Pig	Swine oedema	O.4 : K ? : H.5	—	—	Not done
,,	,, ,,	O.138 : K.81 : H ?	—	—	,, ,,
,,	,, ,,	O.139 : K.82 : H.1	—	—	Mild
,,	,, ,,	O.8. : K? : H.19*	—	—	Moderate
Pig	Swine enteritis	O.4 : K ? : H.5	—	—	Not done
,,	,, ,,	O.8 : K ? : H.19	—	—	Moderate
,,	,, ,,	O.8 : K ? : H.19	—	—	Mild
,,	,, ,,	O.86 : K.61 : H.10	—	—	Not done
,,	,, ,,	O.141 : K.85 : H.4	—	—	,, ,,
Chicken healthy		O.55 : K.59 : H.11	—	—	Mild

H ? = flagella antigen unidentified. K ? = surface antigen unidentified.
* Strain similar to one identified by Dr. F. Ørskov.

Results of tests on strains and extracts of Esch. coli *of animal origin*

Strains of *Esch. coli* of animal origin were tested, the results are shown in Table II. The 2 serotypes O.78 and O.137, commonly found in calf scours, and the rarer type O.26, gave positive results. The 4 unclassified serotypes isolated from calves had been identified by serological methods by Rees (1958), and the same types had been isolated from a number of sick animals. The correlation of strains producing a positive gut test and diarrhoea in the calves from which they were isolated is not as close as in human strains ; this might be due to the strains having been kept in the laboratory for a number of years. None of the strains isolated from either swine oedema or swine enteritis caused dilatation of the rabbit gut. The swine oedema strain O.139 : K.82 : H.1 was isolated by Timoney (1957), who prepared an extract, half of which he injected into a pig, thereby causing swine oedema ; the second half

we injected into a loop of rabbit gut, with negative results. This finding shows that the agent in the extract which caused swine oedema failed to cause dilatation of the rabbit gut. A culture of the living organism was also tested, but failed to cause dilatation. In our previous paper we gave results showing that extracts of strains of *Esch. coli* causing infantile enteritis failed to cause dilatation of rabbit gut, whereas the living strains gave positive results.

The strains from swine enteritis were tested soon after isolation, some within 10 days, but all gave negative results.

The strain from chicken was tested because of its close antigenic similarity to strains causing infantile diarrhoea. Types in the O.55 : K.59 group, commonly isolated in human disease, are non-motile, H.6 and H.7 serotypes, though others also occur. We have identified 3 strains of O.55 : K.59 : H.11, which were isolated from babies, but the clinical history of these cases was not known. These results support the view that the agent causing dilatation of the rabbit gut may be present or absent in strains of identical serotypes.

Results of test on strains of Esch. coli *isolated from water.*

Strains of *Esch. coli* were isolated from farm well waters which were undergoin groutine testing, seven of which were chosen for tests as their somatic antigens were identical with those causing infantile diarrhoea. The strains were one each of O.26 : K.60 : H.32, O.55 : K.59 : H.7 and H.27, O.119 : K.69 : nm., 2 strains of O.127 : K.63 (B.8) : H.4, and one O.128 : K.67 : H unidentified. These strains failed to cause a reaction in the ligated rabbit gut. Serotypes O.26 : K.60 : H.32, O.55 : K.59 : H.7 and O.119 : K.69 : nm. cause infantile diarrhoea. Reference to Table I shows that strains of O.55 : K.59 : H.7 isolated from human cases caused dilatation and inflammation of ligated rabbit gut.

PART 2

Numbers of organisms in ligated gut loops

Results already published showed that large numbers of the *Esch. coli* type which had been injected were present in loops of rabbit gut, but it was suggested that larger numbers might be present in the positive loops, which would account for the reaction. This point was investigated by the following method ; a loop of ligated gut was injected, as described in our previous paper, with 1 ml. of a peptone-water culture containing a known number of organisms. This loop was separated by an uninoculated portion of gut from a second test loop into which 1 ml. of sterile peptone water was inoculated as control ; then another uninoculated portion of gut separated the control loop from the third test loop into which peptone water culture was inoculated. The following day at post-mortem the small gut was removed aseptically. Each test loop was placed in a separate sterile Atomix cup with 100 ml. of sterile normal saline and homogenized. Bacterial counts were made by the Miles and Misra (1938) method. Typical results on 1 of 3 rabbits used are given in Table III, which shows that similar numbers of

TABLE III.—*Number of* Esch. coli *in Loops of Ligated Gut*

Inoculum		Gut	
Serotype	Number injected	Dilatation	Number organisms
O.111 : K.58 : H.12	14×10^3	—	50×10^8
O.26 : K.60 : nm.	6×10^3	+	10×10^8
Peptone water	Sterile	—	3 aerobic sporing organisms

Esch. coli were present in positive and negative loops, and that the loops inoculated with sterile peptone water contained only a few aerobic sporing bacilli ; the latter material was incubated both aerobically and anaerobically. These results prove that the failure of some strains to cause dilatation of the gut is not due to their failure to multiply in that situation.

Effect of colonial variation on results obtained in rabbit gut

We have been investigating *Esch. coli* for a number of years, and noticed that some strains which had been tested many times, and had given positive results for a number of years,

became unreliable in that they gave sometimes positive and sometimes negative results. This effect was observed in strains which appeared to be smooth as well as in those showing smooth-rough change. Some strains, on keeping, show a change to a typical rough colonial form in which the organisms cannot be identified by serological methods. Some strains of *Esch. coli* show a change to a mucoid colony which is of the same serotype as the parent strain, but has an additional mucoid antigen, which was the same in the *Esch. coli* " O " group studied by Taylor and Charter (1955). Smooth, rough and mucoid colony variants were tested. A summary of the results is given in Table IV. It was found that rough variants gave negative results, and that the mucoid variant of a negative strain was also negative. The mucoid variant of O.128 : K.67 : H.2 gave positive results, although the smooth non-mucoid culture was negative.

TABLE IV.—*Effect of Colonial Form of* Esch. coli *on the Gut Test*

Esch. coli		Gut	
Serotype	Colonial form	Dilatation	Histology
O.26 : K.60 : nm.	Smooth	+	Moderate–severe
Variant	Rough	−	Moderate
O.111 : K.58 : H.12	Smooth	−	,,
Variant	Mucoid	−	,,
O.128 : K.67 : H.2	Smooth	−	,,
Variant	Mucoid	+	,,

nm. = non-motile.

Effect of method of storing strain on gut test

Kirby *et al.* (1950) using adult volunteers tested the effect of administering a culture of *Esch. coli* O.111 : K.58 by mouth. The strain used had been isolated from a case of infantile diarrhoea, and had been kept on 2 egg slopes, one stored at 4°, the other at room temperature. They found that symptoms were produced only by the culture which was stored at room temperature. As a result of this observation we decided to test strains which had been stored by different methods. A strain of *Esch. coli* which repeatedly produced a positive reaction was stored at room temperature on Dorset's egg medium, and was freeze-dried by 3 methods ; unfortunately, this Dorset's egg culture was used for other purposes, so a second batch of cultures on Dorset's egg medium were made from the same parent strain as had been used for the preparation of the freeze-dried cultures.

Method 1.—A small amount of 7 per cent glucose broth was added to an over-night culture on an agar slope. This suspension was then freeze-dried.

Method 2.—Culture freeze-dried in 5 per cent Evans' peptone + 5 per cent glucose.

Method 3.—Culture was pre-freezed at −40° in 5 per cent Evans' peptone.

Method 4.—Culture was freeze-dried in mist desiccans, Fry and Greaves (1951).

The cultures were kept at room temperature for 9 months. The ampoules opened and a few drops of peptone water mixed with the material which was then transferred to 4 ml. of peptone water, incubated at 37° for 24 hr. Peptone water was added to this culture until the opacity matched the standard, then a standard loop used to transfer one loopful to 4 ml. of peptone water, 1 ml. was injected into the gut loop. Material was taken from the Dorset's egg slope and inoculated into peptone water ; this culture was treated in a similar manner. Tests were repeated three times, the results are shown in Table V. A culture of *Esch. coli* O.55 : K.59 which had been freeze-dried in mist desiccans and stored at room temperature was also tested. Freeze-drying methods may have reduced the ability of *Esch. coli* to produce a positive test, though results after 2 and 7 years storage were quite good.

Effect of medium on the ability of Esch. coli *to produce a positive gut test*

Many of these tests were made with D.5301, a strain of *Esch. coli* O.128 : K.67, as it had been found that the results were sometimes positive, sometimes negative. The method used was to subculture daily, except at weekends, in the medium under test. Usually, the strains

TABLE V.—*Effect of Freeze-drying Cultures on Gut Test*

Number of rabbits	Serotype	Culture	Period	Gut	
				Dilatation	Histology
2	O.26 : K.60 : H–	Freeze-dried (1)	9 mth.	–	Mild–moderate
		,, ,, (2)	9 ,,	–	Moderate
		Egg slope	6 ,,	+	,,
1	O.26 : K.60 : H–	Freeze-dried (1)	9 ,,	+	Mild
		,, ,, (2)	9 ,,	+	Moderate
		Egg slope	6 ,,	+	,,
1	O.26 : K.60 : H–	Freeze-dried (2)	2 yr. 4 mth.	+	,,
		,, ,, (3)	2 yr. 4 mth.	+	,,
		Egg slope	1 yr.	+	Mild–moderate
1	O.55 : K.59	Freeze-dried (4)	7 ,,	+	Moderate
		Egg slope	6 ,,	+	,,

Figures in brackets indicated method of culture storage (see text).

were tested after about 10–15 passages, when the organism was grown in the test medium for 18 hr. If the medium itself were transparent, then the culture was diluted with the same medium to standard opacity; the volume added was used as a guide for the dilution of opaque media. A new tube of the test medium was inoculated with a standard loopful of diluted culture and 1 ml. of this was inoculated into the loop. Bacterial counts were made on all inocula. The media tested were cooked meat (veal) broth, tested after aerobic and anaerobic incubation, 5 per cent horse blood in nutrient broth, 2 per cent hog gastric mucin in peptone water, 5 per cent human ovomucin in peptone water, cow's milk. In each instance the sterile medium was tested and failed to cause dilatation or exudate or to affect the histological findings. The results given in Table VI suggest that 2 findings might be important, firstly

TABLE VI.—*Effect of Media on Strains Used in the Gut Test*

Strain	Medium	Total number tests	Results		Histology
			Number dilated	Number normal	
D.5301	Peptone water	9	3	6	Moderate
	Cooked meat (aerobic)	3	1	2	Moderate–severe
	,, ,, (anaerobic)	1	0	1	Mild–moderate
	Ovomucin	1	0	1	,, ,,
	Gastric mucin	4	0	4	Moderate
	5 per cent horse blood broth	3	2	1	,,
	Milk	4	4	0	Moderate–severe
D.2101	Peptone water	100	0	100	Mild
	Milk	2	0	2	Very mild
E.65	Peptone water	100	100	0	Moderate
	Gastric mucin	2	2	0	,,

that milk enhances the pathogenic effect, and secondly that hog gastric mucin reduces the effect. The details of the 2 experiments show these effects particularly clearly (Table VII). Both these rabbits gave typical gut reactions with the control strain E.65. Rabbit 95 gave a strongly positive reaction with the peptone water culture of D.5301, but failed to react to the same organism in hog gastric mucin. Rabbit 97 failed to give a positive test with a peptone water culture, but gave a good positive reaction with the milk culture.

D.2101 which gave consistently negative results by the usual method was tested after 9 and 18 passages in milk; the results were unchanged.

TABLE VII.—*Effect of Media*

Rabbit No.	Strain	Medium	Number organisms injected	Gut	
				Dilatation	Histology
95	D.5301	Gastric mucin	51×10^{11}	—	Moderate
		,, ,,	O	—	Mild
	D.5301	Peptone water	20×10^{6}	+	Moderate
	E.65*	,, ,,	18×10^{6}	+	,,
97	D.5301	Milk	14×10^{5}	+	Moderate–severe
		,,	O	—	Mild
	D.5301	Peptone water	5×10^{5}	—	Moderate
	E.65*	,, ,,	4×10^{3}	+	Moderate–severe

* E.65 = control positive strain of *Esch. Coli.*

E.65, which always gave positive results, was tested after 9 and 13 passages in hog gastric mucin; the results were unchanged.

D.5301 was used to find out whether the enhancing effect of growth in milk was reversed by changing to hog gastric mucin; also whether the depressing effect of growth in hog gastric mucin was reversed by changing to milk. The method used was to subculture serially D.5301 in 2 per cent hog gastric mucin 6 times (primary medium) from an 18 hr. culture in mucin a loopful was inoculated into 4 ml. of sterile milk, 1 ml. of this was injected into the gut loop; the remaining 3 ml. of the milk were incubated for 18 hr. and one loopful transferred to another tube of milk (secondary medium), 1 ml. of which was inoculated into the gut loop, the rest being incubated, and tests continued in a similar manner on further serial subcultures. The results are shown in Table VIII. These results show that the negative results of the strain,

TABLE VIII.—*Reversal of Effect of Media, Strain D.5301*

Primary passage medium	Secondary passage medium	Number organisms injected	Gut		
			Dilatation	Exudate	Histology
Mucin (6)	—	13×10^{4}	—	—	Mild–moderate
	Milk	12×10^{4}	+	+	,, ,,
	,, (1)	5×10^{4}	—	—	,, ,,
	,, (11)	3×10^{4}	+	+	Moderate
Mucin (11)	—	8×10^{4}	—	—	Mild–moderate
	Milk	7×10^{4}	Trace	—	Mild
	,, (1)	2×10^{4}	+	+	O
	,, (2)	5×10^{4}	+	+	Moderate–severe
	,, (4)	4×10^{4}	+	+	O
Milk (6)	—	31×10^{4}	+	+	Severe
	—	7×10^{4}	+	+	Moderate–severe
	Mucin (1)	7×10^{4}	—	—	Mild–moderate
	,, (11)	9×10^{4}	—	—	Moderate
Milk (11)	—	15×10^{4}	+	+	Moderate–severe
	—	7×10^{4}	+	+	Moderate
	Mucin (1)	3×10^{4}	Trace	Trace	O
	,, (2)	10×10^{4}	+	+	Moderate–severe
	,, (4)	5×10^{4}	—	—	O

O = not done.
Figures ( ) = number of serial subcultures.

when inoculated in gastric mucin, become positive when the medium is changed to milk; also that the reverse change occurs. The reversal in the gut effect is more rapid with a change from mucin to milk medium, as in one experiment the inoculation of a mucin-passaged strain, when inoculated in milk, gave a positive result in the ligated gut, whereas a milk-passaged strain needed to be cultured usually twice in mucin before a negative result was obtained.

Effect of active and passive immunization on response in ligated loop

Active immunization.—Rabbits were immunized with living suspensions of *Esch. coli* O.26 : K.60. The organism was grown on 0·1 per cent glucose in nutrient agar, the growth harvested in ¼ strength Ringer and diluted to standard opacity indicating 500 × 10^6 organisms per ml. Starting with a first intravenous injection of 0·1 ml. the volume was doubled on each succeeding occasion until a final dose of 1·6 ml. was given at the 5th injection. The interval between doses was 2 or 3 days. A test bleeding was made from each rabbit 5 days after the last injection. the titres were O = 6400–12,800, K = 400 in each rabbit. Between 5–7 days after the last injection, challenge inoculations were made using the strain used for immunization and strains of unrelated serotypes which had given positive results in ligated gut loops. At the same time the cultures were tested on a normal rabbit as a control.

Five rabbits were immunized with E.65, *Esch. coli* O.26 : K.60 ; in each a loop of gut was injected with a culture of E.65, 3 tests were negative, 2 positive ; the control normal rabbit gave positive results with the 5 cultures. These results show that immunization gave some protection against the action of the *Esch. coli* used for immunization. Rabbits which had been immunized with *Esch. coli* O.26 : K.60 were tested with *Esch. coli* serotypes O.111 : K.58 and O.128 : K.67. The results were not very satisfactory, but there was no protection against the action of serotypes different from the immunizing strain.

Passive immunization

The effect on rabbit gut of injecting known positive strains and antiserum was tested. Cultures were grown in the usual way for 18 hr., and diluted to standard opacity. Two standard loopfuls were transferred to a mixture of 1 ml. of test serum and 1 ml. peptone water ; 1 ml. of mixture was used for inoculation, and bacterial counts were made on the remainder. Controls were made by inoculating a mixture of normal rabbit serum with the test strain. The results are given in Table IX, which shows that O.26 : K.60 antiserum protected the

TABLE IX.—*Effect of Inoculating Mixtures of Immune Sera and* Esch. coli

Inoculum					Gut	
Serum			Organism			
Immunizing strain	Type	Titre	Strain	Type	Dilated	Histology
Nil	Normal rabbit serum	*Nil*	E.65	O.26 : K.60	+	Moderate
			G.193	O.55 : K.59	+	,,
			E.177	O.119 : K.69	+	Mild–moderate
E.65	O.26 : K.60	O = 12,800 K = 1,600	E.65	O.26 : K.60	−	Mild
			E.854	O.26 : K.60	−	Very mild
			G.193	O.55 : K.59	+	Moderate–severe
Aberdeen β	O.55 : K.59	O = 6,400 K = 800	G.193	O.55 : K.59	−	Moderate
E.3729	O.119 : K.69	O = 3,200 K = 200	E.177	O.119 : K.69	−	Mild

gut from the action of cultures of *Esch. coli* O.26 : K.60, but had no effect on cultures of other *Esch. coli* serotypes, and that similar results were obtained with other serotypes and the corresponding specific sera. It was thought that these results might be influenced by the ability of the organism to grow in serum mixtures injected into the gut. To investigate this point, bacterial counts were made on the loops after removal by the method described earlier. The results shown in Table X demonstrated that *Esch. coli* increased in numbers to about the same degree when inoculated with the homologous antiserum, with a heterologous antiserum, or without the addition of serum ; also that the same number of organisms were present in the positive and negative loops.

TABLE X.—*Numbers of* Esch. coli *in Mixtures with Antisera*

Rabbit	Serum	Inoculum: Strain	Inoculum: Serotype	Inoculum: Number injected	Gut: Dilatation	Gut: Total number *Esch. coli*
141	—	D.2101	O.111 : K.58 : H.12	11×10^3	—	13×10^8
	O.26 : K.60	E.65	O.26 : K.60	21×10^3	—	2×10^8
	O.26 : K.60	—	—	—	—	—
	—	E.65	O.26 : K.60	19×10^3	+	19×10^8
144	—	D.2101		19×10^3	—	11×10^8
	O.26 : K.60	G.193	O.55 : K.59	4×10^4	+	36×10^8
	—	G.193		24×10^3	+	11×10^8
	—	E.65		16×10^3	+	31×10^8

DISCUSSION

For many years it has been recognised that there is a need for some method for testing the ability of an organism to cause diarrhoea. A number of different enterobacteria have been tested in human volunteers, and valuable results obtained. Strains of *Esch. coli* have been tested in babies by Neter and Shumway (1950), and in adults by Kirby, Hall and Coackley (1950) and Ferguson and June (1952), but it is difficult to get human volunteers. In addition, it is impossible to determine their immunity to a particular organism before carrying out the experiment. The ligated rabbit technique seemed to give results which could be correlated with the ability of an organism to cause diarrhoea in man. It seemed important to find out if this test could be usefully applied to organisms believed to cause diarrhoea in animals, also if *Esch. coli* causing diseases not associated with diarrhoea also produced a positive test. In other words, was this a test for pathogenicity in general or a test for enteropathogenicity? We believe that the latter is true, as, using strains of *Esch. coli* isolated from cases of infantile diarrhoea, in which the organism was believed to be the aetiological agent, good correlation was found with the ability to cause dilatation of the ligated loop of gut, whereas, using similar serotypes isolated from healthy babies, healthy animals, or well water, the results were negative. *Esch. coli* causing human urinary infections, swine oedema and swine enteritis, gave negative results, but some strains causing calf scours gave positive results. The correlation between a positive gut test and an aetiological role in animal diarrhoeal diseases was not close.

The reaction of a doubtful strain is affected by the medium in which it is grown, and in which it is inoculated into the ligated gut, milk enhancing the reaction, whereas hog gastric mucin caused a negative reaction. With a strain giving strongly positive reactions the hog gastric mucin is without effect. This finding may be related to the treatment of infantile diarrhoea, as it has been found that water only should be given by mouth until the symptoms improve.

Rabbits were actively immunized with living cultures of *Esch. coli* injected intravenously. Some rabbits were protected, and failed to give a positive test, others gave the usual positive result. Although the experiments were rather unsatisfactory there appeared to be no protection against challenge with heterologous serotypes. By injecting mixtures of positive strains with the homologous antiserum, there was complete protection, but no protection against other serotypes. This finding agrees with the findings in human infection, where babies recovering from diarrhoea due to one serotype became infected with a different serotype and developed another bout of diarrhoea (Anderson, Crockatt and Ross, 1954).

SUMMARY

Ligated loops of rabbit gut have been used to test strains of *Esch. coli* for their ability to cause dilatation. It was found that strains which caused infantile enteritis caused dilatation, but strains causing human urinary infection were without effect. As far as human strains are concerned, a positive gut test is not a test for general pathogenicity, but a test for the ability of the organism to cause diarrhoea, probably as the result of its ability to produce some toxic substance. Some strains isolated from calf scours caused dilatation, but the correlation was not absolute—possibly because some had been kept in subculture for years—possibly because the pathological processes in this disease, which is accompanied by septicaemia, are different from those in infantile enteritis, in which the infection is localised to the gut. Strains of *Esch. coli* associated with swine enteritis and swine oedema were without effect, as were *Esch. coli* serotypes which cause infantile diarrhoea, but which had been isolated from well water.

The effect of colonial variation was investigated. It was found that smooth strains of *Esch. coli* isolated from infantile enteritis caused dilatation of the ligated loop, whereas the rough variant was negative. Some smooth strains of *Esch. coli* gave variable results ; with such cultures the mucoid variant gave a positive reaction.

The effect of media was tested. Strains which gave variable results when injected in peptone water, gave positive results when injected in milk, but negative results when injected in 2 per cent hog gastric mucin in peptone water. Media had no effect on strains which invariably gave positive results, or those which gave negative reactions. The same number of organisms was found in positive and negative loops.

Active immunization of the rabbit gave partial protection, passive immunization gave complete protection, against the homologous serotype, but no protection against different serotypes.

We wish to thank Dr. Joan Stokes for strains of *Esch. coli* from human urinary infections, Professor R. Lovell for strains from calf scours, Mr. J. M. Leach for strains from swine enteritis, Mr. J. F. Timoney and Miss R. Lemcke for strains from swine oedema, and Mr. G. Elis Jones for strains from well water.

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SEROLOGICAL RESPONSES TO ASIAN INFLUENZA VIRUSES IN MAN: A COMPARISON OF HAEMAGGLUTINATION-INHIBITION AND COMPLEMENT-FIXATION METHODS

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THE serological response to Asian influenza infection demonstrated in man by haemagglutination-inhibition tests appears to be virtually strain-specific in character and limited to the antigen of the Asian (A2) influenza virus (Menon, 1959 ; Zhdanov and Ritova, 1959). Henle, Lief and Fabiyi (1958) showed, however, that complement-fixation techniques with purified strain-specific virus particle (V) antigens (Lief and Henle, 1956*a* and *b*) detected significant antibody responses not only to the homologous infecting virus but to V antigens derived from a variety of strains of influenza virus from the A and A1 families. Further information therefore was sought concerning the range of antibody responses resulting from exposure to Asian influenza virus in Britain by a comparison of haemagglutination-inhibition (HI) tests and complement-fixation tests using virus particle antigens (V-CF tests). The two methods were first compared in tests with antisera from animals and then with sera from two groups of persons. The first group consisted of 25 healthy volunteers (naval recruits aged 20–30) drawn from a larger group inoculated with a monovalent inactivated Asian virus vaccine in the spring of 1959 (Himmelweit, unpublished). In the second group paired sera from 50 cases of influenza occurring between September 1957 and March 1959 were selected on a basis of positive virus isolation or of fourfold or greater rise in antibody to influenza virus soluble antigen (Type A). There was no selection by age in the latter group ; the 50 cases ranged from 8–75 yr.

From each person in the 2 groups paired sera were taken before and after the period of exposure to the virus antigen. In all such pairs, the pattern of antibody response to each of the main families of influenza A viruses was estimated separately, first by HI tests and secondly by V-CF tests.

MATERIALS AND METHODS

Sera

All specimens were kept frozen at — 20°. Convalescent ferret antisera were prepared by intranasal inoculation of infected allantoic fluids (WHO Report, 1953). Guinea-pig "antisoluble" antiserum (anti-S) was produced by intranasal inoculation with live virus of strain WS followed by intraperitoneal injection of "soluble" (S) antigen prepared from PR8 virus (Lief, Fabiyi and Henle, 1958). All human sera were paired specimens ; in the vaccinated group samples were taken one week before and 3 weeks after inoculation ; in the group of clinical cases of influenza, samples were taken when the patient was first seen in the acute phase, and 10–14 days later during convalescence.

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Viruses

The strains of influenza used were Swine, WS and PR8 representative of the family A, FMI as typical of the family A1 and Pakistan I (kindly provided by Dr. F. Himmelweit, the Wright-Fleming Institute of Microbiology, London) as a member of the Asian family. All virus preparations were stored at − 70° until use.

Pools of infected allantoic fluids for HI tests were prepared in eggs by standard methods (WHO Report, 1953). The tests were performed by a modification of the plastic plate technique (WHO Report, 1953) using 0·02 M-phosphate buffered saline at pH 7·0 and a 0·5 per cent suspension of hen red blood cells. Sera for HI tests were treated with commercial cholera filtrate (Philips-Roxane) to remove non-specific inhibitors. Virus suspensions were used at a concentration of 8 haemagglutinating units per test volume.

V antigens were prepared from infected allantoic fluids by 2 cycles of adsorption on to and elution from fowl red blood cells (Lief and Henle, 1956*b*). V-CF tests were performed by a single-line modification of the microdrop method of Fulton and Dumbell (1949) with a fixation period of 1½ hr. at 37°. Sera were inactivated by heat at 56° for 30 min. before testing. Each V antigen was used at an optimum dilution determined by chessboard titrations with known positive ferret antisera.

S antigen was prepared from chorio-allantoic membranes (WHO Report, 1953) using the PR8 virus, and the test procedure was exactly similar to that with V antigens.

In both CF and HI tests antibody titres were expressed in terms of the initial dilution of serum.

RESULTS

Tests with animal antisera

Comparative titrations were performed by HI and V-CF tests with materials prepared from each of the 5 standard virus strains and with S antigen; results are shown in Table I.

TABLE I.—*Comparison of Haemagglutination-inhibition (HI) and Complement-fixation (CF) Titres in Sera from Animals Immunized Against Specific Influenza A Strains.*

			Antisera				
			Ferret				
Test	Antigen		WS	PR8	FMI	Asian	Guinea-pig anti-S
HI	Allantoic fluids	Swine	0*	0	0	0	0
		WS	*128*†	4	0	0	0
		PR8	4	*128*	0	0	0
		FM1	0	0	*256*	0	0
		Asian	0	0	0	*512*	0
CF	Purified V antigens	Swine	0	0	0	0	0
		WS	*256*	0	0	0	4
		PR8	4	*128*	0	0	4
		FM1	0	0	*512*	0	0
		Asian	0	0	0	*256*	0
	Soluble antigen		128	256	256	128	64

* 0 = no detectable reaction at 1/4 dilution of serum.

† Titres expressed as denominator of dilution. Titres of sera with homologous immunizing virus are in italics.

In each of the 4 ferret antisera, HI antibody was demonstrated only against the homologous immunizing strain with the exception of a very weak reciprocal cross-reaction between WS and PR8. These sera also contained significant and

comparable amounts of CF antibody to the shared soluble (S) antigen. The guinea-pig antiserum contained only anti-S antibody.

The panel of V antigens gave strain-specific reactions with the ferret antisera and titres were closely similar to those obtained in HI tests. There were no heterologous cross-reactions of significance despite the presence of anti-S antibody in all 4 sera ; with the guinea pig anti-S serum, only trace reactions were found confined to the 2 strains used for immunization.

These results suggest that the V antigen preparations were essentially free from detectable S antigen and were capable of demonstrating a strain-specific response to immunization similar to that shown by HI tests. Accordingly, the 2 groups of human sera were similarly investigated with the same batches of reagents.

Tests with sera from vaccinated persons

The pattern of serological response to Asian vaccine is shown in Figs. 1 and 2*a* in which the distribution of the various antibodies in the sera before vaccination is compared with the distribution 3 weeks after immunization.

HI tests.—With the homologous Asian antigen (Fig. 1*a*) a marked rise in antibody was found ; 15 (60 per cent) of the prevaccination sera were negative (titre $<1/4$) and the remainder (40 per cent) showed titres of 1/16 or less, whereas 24 (96 per cent) of the post-vaccination samples were positive and in 18 cases (72 per cent) titres were 1/128 or greater.

All the prevaccination sera contained some antibody to FMI and 13 (52 per cent) were also weakly positive against PR8 antigen ; none were positive with Swine antigen (Fig. 1*a*). In no individual, however, was there a significant (*i.e.* 4-fold or greater) rise in antibody to these strains and the post-vaccination distribution of HI antibodies to all the various viruses was essentially the same as before immunization.

CF tests.—The distribution of antibodies found with V antigens in the same 25 pairs of sera is shown in Fig. 1*b*. With Asian V antigen, a significant rise in antibody was found as with the HI tests. Most prevaccination sera were negative or only weakly positive, but 24 (96 per cent) of the postvaccination samples were positive and 18 (72 per cent) of cases had titres of 1/128 or greater.

With the 4 V antigens from the other strains some antibody was found to one or more antigens in the prevaccination sera (Fig. 1*b*). However, no significant change in the distribution of antibody to any of these earlier strains occurred as a result of immunization.

With S antigen (Fig. 2*a*) only 7 (28 per cent) positive reactions were found in postvaccination specimens and in no case was the titre greater than 1/8.

The overall pattern of results suggests that the only significant response to Asian vaccine was a rise in antibody to the Asian strain and both HI and V-CF tests appeared to show this marked response with equal efficiency (Table II). It may be noted, however, that the lower stationary levels of antibody to viruses recovered before the Asian epidemic were not detected equally well by both methods and both CF positive, HI negative and CF negative, HI positive sera were found.

Tests with sera from cases of influenza

HI tests.—The development of antibody directed against the Asian strain (Fig. 3*a*) was less striking than in the group of vaccinees (Fig. 1*a*) ; 20 cases (40

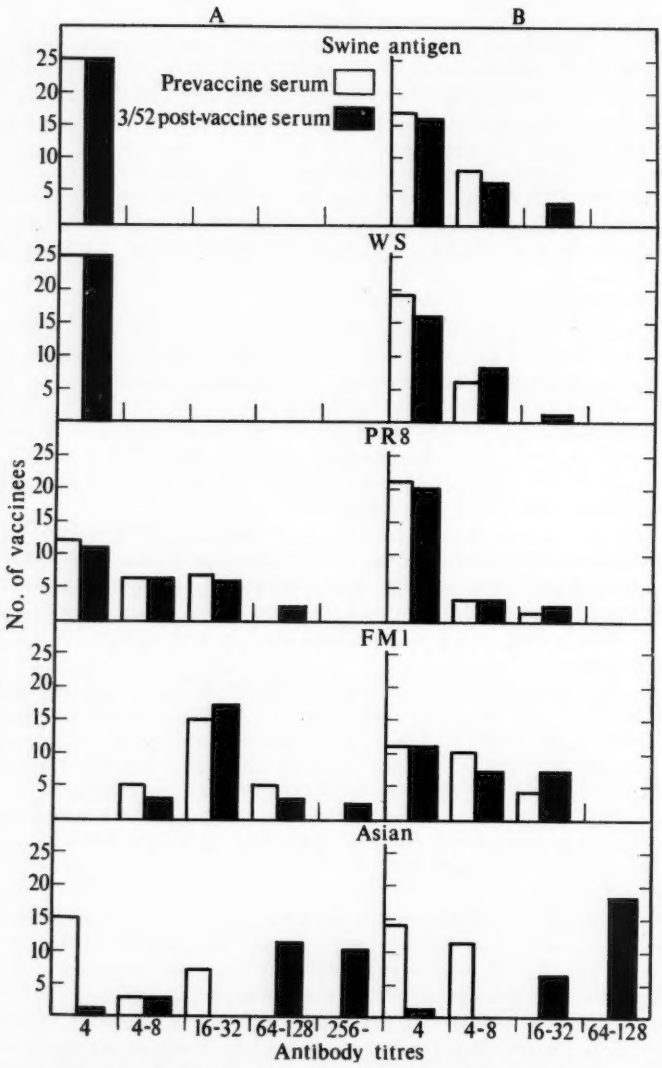

FIG. 1.—Changes in the distribution of antibody titres in man resulting from vaccination with A/Asian strain of influenza virus. Comparative titrations of paired sera from 25 volunteers with haemagglutinating and complement-fixing antigens of 5 influenza A strains.

A = haemagglutination inhibition tests.
B = virus (particle antigen) complement fixation tests.

Antibody titres labelled 4 in fact represent values of <4.

per cent) showed a 4-fold or greater difference in titre between the acute phase and convalescent samples of serum.

HI antibodies to one or more of the other virus strains were found in several cases in both samples (Fig. 3*a*). The distribution of titres to these strains changed

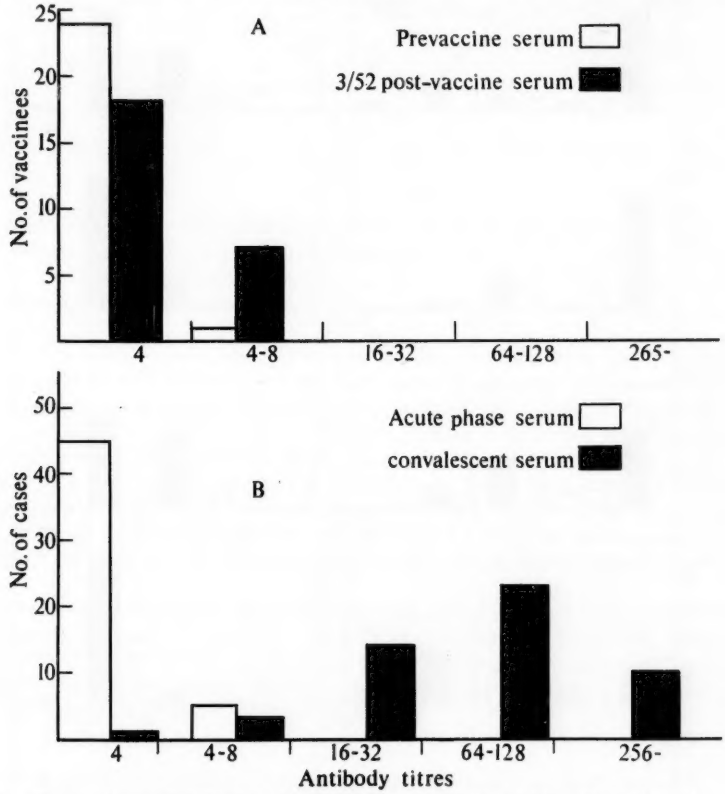

FIG. 2.—Changes in the distribution of antibody to influenza A soluble antigen in man.
A = following vaccination with killed Asian virus.
B = following clinical influenza September, 1957–March, 1959.
Antibody titres labelled 4 in fact represent values of <4.

to only a limited extent as a result of infection ; with Swine strain, 8 patients (16 per cent) developed a 4-fold or greater rise in titre between the 2 serum samples. In general however the pattern suggested that the predominant response to Asian virus infection was a rise in strain-specific anti-Asian antibody (Table II).

CF tests.—The distribution of antibodies to V antigens of the 5 virus strains in the same 50 pairs of infective sera is shown in Fig. 3*b*. Of the acute phase specimens, 41 (82 per cent) did not contain detectable antibody to any of the V antigens ; in the remaining 9 acute phase sera, only one contained detectable antibody to Asian antigen, but low titres (1/4–1/16) of antibody to one or more of the other virus

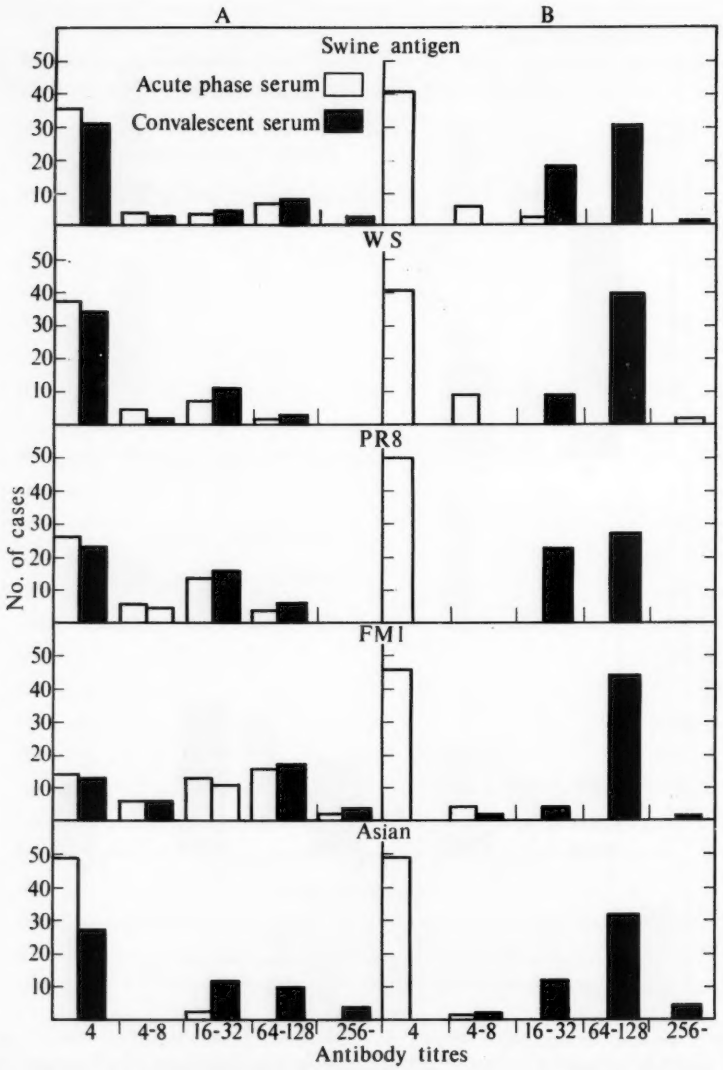

FIG. 3.—Changes in the distribution of antibody titres resulting from influenza infection in 50 cases (September, 1957–March, 1959). Comparative titration of paired sera with haemagglutinating and complement-fixing antigens of 5 influenza A strains.

A = haemagglutination inhibition tests.
B = virus (particle antigen) complement fixation tests.

Antibody titres labelled 4 in fact represent values of <4.

TABLE II.—*The Effect of Exposure to A/Asian Influenza Virus on Antibody Levels to Various Type A Virus Strains in Man.*

Source of human sera	Percentage of serum pairs with > 4-fold rise in antibody titre										
	HI					CF Soluble antigen	CF V antigens				
	Swine	WS	PR8	FM1	Asian		Swine	WS	PR8	FM1	Asian
Vaccination with A/Asian strain (25 cases)	0	0	0	0	92	4	8	0	4	0	92
Clinical influenza infections (50 cases)	16	8	4	10	40	96	100	100	100	100	98

strains were found. As with the group of vaccinated persons these low resting levels of pre-Asian antibodies were not detected with equal efficiency by V-CF and HI tests ; the greatest discrepancy was in the case of the FMI strain where 36 (72 per cent) acute phase sera were found positive by HI tests, but only 4 (8 per cent) by V-CF tests.

In the convalescent sera, the pattern of results obtained with the 5 V antigens differed sharply from V-CF results with post-vaccination sera. All the convalescent sera gave a reaction to high titre with every antigen regardless of the strain from which the antigen had been derived (Fig. 3*b*). Thus in contrast with the pattern found in HI tests with the same serum pairs (Fig. 3*a* and Table II) there was no evidence of any greater response to the homologous Asian strain than to any of the 4 heterologous antigens. With each V reagent the distribution of antibodies changed from an acute-phase pattern of less than 12 per cent positive at 1/4 to a convalescent pattern of 100 per cent positive at 1/32 or more with average titres of 1/64.

In CF tests with S antigen (Fig. 2*b*) there was also a marked change in antibody distribution as a result of infection. Only 5 (10 per cent) of the acute phase sera were positive at a titre of 1/4, whereas 49 (98 per cent) of convalescent specimens were positive with an average titre of 1/64. This finding was to be expected in view of the selection of cases mentioned earlier.

DISCUSSION

The results obtained with haemagglutination-inhibition tests in the present investigation were similar to those described by other workers in cases of Asian influenza (Menon, 1959 ; Zhdanov and Ritova, 1959) or following vaccination with monovalent Asian preparations (Himmelweit, unpublished). They differed in many respects, however, from the serological response to influenza viruses observed before 1957, when it had repeatedly been shown that rises in HI or neutralizing antibody titre following influenza A or A1 infections not only embraced strains other than the homologous infecting strain (Stuart-Harris, Andrewes and Smith, 1938), but might actually be greater for heterologous viruses than for the current epidemic virus (Stuart-Harris and Miller, 1947). With increasing age the reactivity of such sera became increasingly broad, and each successive exposure to a type A or A1 strain by natural infection (Davenport, Hennessy and Francis, 1953 ; Davenport, Stuart-Harris, Hennessy and Francis, 1955 ; Hennessy, Davenport

and Francis, 1955), or by vaccination (Davenport and Hennessy, 1956 ; Jensen, Davenport, Hennessy and Francis, 1956) appeared to stimulate an immunological recall of antibodies resulting from previous infections. Commonly, the highest antibody titres were those against strains first encountered in childhood.

In the present investigation HI antibodies to one or more of the A and A1 virus families were found in sera collected before the exposure of their donors to Asian virus. In the prevaccination samples, all were positive to FMI and 13 of the 25 contained antibody to PR8 ; no antibodies to Swine or WS strains were detected in this group. In the preinfective samples FMI antibodies were less predominant, and almost one-third of all samples contained antibody against PR8, WS and Swine strains, the latter being restricted to those over 35 yr. old. The serological differences between the 2 groups were probably due to differences in the age-structure of the 2 groups and are similar to those found by Davenport *et al.* (1955).

Regardless of age the HI antibody response to Asian virus exposure was remarkably narrow in both groups. The vaccinated individuals all showed a significant rise in HI antibody to the homologous antigen but in no case was there an accompanying rise in antibody to any other strain. In the group of clinical infections, 22 of the 50 cases showed a rise in anti-Asian HI antibody irrespective of the age of the individual concerned. In 8 of these 22 cases, all over 35 yr. old, there was an accompanying rise in pre-existing anti-Swine HI antibody, but in no case did anti-Swine or other heterologous antibody arise *de novo* following infection, and the increase in antibody titre to the homologous Asian antigen was consistently greater than that to Swine antigen.

Thus there is only slight evidence that the recall phenomenon described in earlier epidemics is operative after exposure to Asian viruses. Similarly, direct examination of Asian strains (Menon, 1959 ; Fukumi, 1959) has so far failed to demonstrate in them any of the haemagglutinating antigens known to be shared widely amongst A or A1 strains (Jensen and Francis, 1953) and presumably responsible for the broad spectrum of HI antibody response to these earlier viruses.

The results obtained with the present groups of human sera in V-CF tests differed in several important respects from the HI test results discussed above. In preliminary standardization experiments with animal antisera it was apparent that HI and V-CF tests were each capable of detecting with comparable efficiency a strain-specific response to infection and that the specificity of the V antigens was not obscured by the presence of antibody to the shared S antigen. These results were as would be expected from the basic studies of Henle and his colleagues in this field (Henle and Wiener, 1944, Lief and Henle, 1956*a* and *b* ; Lief, Fabiyi and Henle, 1958 ; Fabiyi, Lief and Henle, 1958 ; Lief, Ostapiak, Fabiyi and Henle, 1958 ; Lief and Henle, 1959).

In the two human groups, however, " resting " levels of antibody to the various earlier (pre-Asian) viruses in the first serum specimens were not detected with equal efficiency by the two methods, and particularly in the case of FMI several sera found positive by the HI test were negative in V-CF tests with antigens derived from the same initial virus seed.

In the vaccinated group, all cases showed a significant rise in CF antibody to the Asian V antigen but in no case was there an accompanying rise or induction of antibody to S antigen or to any of the pre-Asian virus preparations. In pattern, therefore, the actual response of this group to immunization was similar whether judged by HI or by V-CF tests.

In the group of influenza infections, however, the result of Asian virus exposure as judged by V-CF tests was strikingly different from that shown by HI tests in the same set of sera, and contrasted with the pattern of response following vaccination. Few acute-phase sera contained demonstrable antibody to any of the V antigens, whereas in each of the 50 convalescent specimens antibody titres against all these 5 reagents had risen significantly. Antibody titres detected by antigenically-remote strains such as Swine and WS were as high as those shown by the Asian antigen, even in individuals too young ever to have been exposed to infection by such early type A strains. In general, the magnitude of the response to V antigens in these serum pairs seemed comparable to that shown to the shared S antigen; the pattern of response appears to be as non-specific as that obtained by Lennette, Culver and Stevens (1958) with crude egg-fluid antigens. This spectrum of reactivity to V antigens shown in the present investigation could not be attributed to cross-reactions with anti-S antibody in view of the satisfactory behaviour of the same virus reagents with animal sera.

These results suggest that Asian strains of influenza viruses share certain particle complement-fixing antigens in common with earlier A and A1 strains, apart altogether from the shared soluble S antigen. Limited evidence of such relationships have also been demonstrated by direct antigenic analysis with standard specific anti-V sera (Henle *et al.*, 1958), but it is clear that these shared antigenic components are not detected by HI techniques.

In single experimental infections of laboratory animals or in human vaccination with killed virus preparations it would appear that the main immunological response is to major antigens which are effectively strain-specific. This response can be detected equally well by HI or V-CF techniques. Under these artificial conditions of exposure, it would appear that the minor shared particle components are not immunologically active enough to produce detectable antibody.

Under conditions of natural infection in man, however, where antigenic release is prolonged, the antibody response to a given virus strain would be less likely to be restricted to the predominant antigen. Broadly-reactive antibodies directed against antigens shared with other type A viruses would also be induced.

Each subsequent infection with succeeding virus strains would recall previous experience of the same common components. The reactivity of such sera with virus particle suspensions *in vitro* would thus emphasize common factors in V preparations rather than the major antigen characterizing the particular strain, and the V-CF antibody response would become comparable in speed and in degree to the response shown by S antibody, as in the present experiments.

This hypothesis is in keeping with 2 interesting observations of Henle *et al.* (1958). Firstly these authors found that repeated immunization of laboratory animals with a single strain of live virus produces a progressive departure from the apparent strain-specific response to a single inoculation; ultimately, a wide spectrum of anti-V antibodies could be found, often reacting with influenza A strains in which no antigenic sharing could be demonstrated by direct analysis. Secondly, in contrast to their findings with live virus, Lief and her colleagues showed that animals inoculated repeatedly with non-infectious haemagglutinating preparations (probably analogous to vaccine materials) continued to produce only strain-specific antibody responses.

The present experiments show that exposure to Asian viruses yields a pattern of serological response which varies according to the nature of the virus exposure,

and according to the method of *in vitro* examination. As judged by HI tests there is little evidence that Asian viruses can stimulate immunological recall of former A or A1 antibodies, yet recall is clearly suggested by the results of V-CF tests.

It remains to be shown whether the particle antigens which are operative in V-CF tests are necessarily identical to those concerned in haemagglutination-inhibition and whether the anti-V responses demonstrated are correlated in any way with immune resistence to infection.

SUMMARY

Purified particle (V) antigens of A, A1 and Asian influenza viruses gave strain-specific reactions in CF tests with animal antisera.

With human sera from healthy volunteers given monovalent Asian vaccine comparable rises in Asian specific antibody were demonstrated by haemagglutination-inhibition and by complement-fixation with V antigens.

With sera from cases of Asian influenza the antibody response detected by V antigens was not strain specific and was not comparable with HI results in the same group.

The significance of these findings in relation to possible antigenic similarities between Asian and pre-Asian influenza A strains is discussed.

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COMPETITIVE TOLERANCE OF SKIN HOMOGRAFTS IN ADULT SEPARATED RAT PARABIONTS

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BLOOD cells exchanged between parabionts after the establishment of vascular anastomoses may survive and cause "parabiotic disease", a fatal condition ascribed to production of antibodies by transplanted blood cells (Nakić, Nakić and Silobrčić, 1960). The similarity between "parabiotic disease" and other conditions such as "runt disease" (Billingham and Brent, 1957) and "secondary disease" (Ilbery, Koller and Loutit, 1958) has already been pointed out (Nakić and Silobrčić, 1958; Nakić *et al.*, 1960). All hosts unable to destroy the graft from their partner and weighing 160 g. or less die of this disease. An analysis of results reported previously revealed a statistically significant positive correlation between survival time of tolerant hosts and their body-weight (age). We decided, therefore, to perform a series of experiments on adult animals. An interesting result was obtained indicating that tolerance to homografts is not necessarily connected with fatal manifestations of "parabiotic disease" and that in adult parabionts the lethal outcome is not a *sine qua non* of tolerance. We also intended to find out whether by varying the duration of parabiotic union an increase in percentage of tolerant animals could be obtained.

MATERIALS AND METHODS

Albino rats of both sexes, weighing 140–230 g. from 2 genetically unrelated random-bred strains, Y and W, were used. The partners always belonged to a different strain and were of the same weight and sex. As already reported (Nakić and Silobrčić, 1958) the normal mean survival times of free skin grafts transplanted from W to Y rats and *vice versa* are as follows: $W \rightarrow Y$, $8{\cdot}1 \pm 0{\cdot}4$; $Y \rightarrow W$, $10{\cdot}2 \pm 0{\cdot}4$ days. No graft survives beyond the 12th day.

The animals were joined in parabiotic union by a modification of the technique of Bunster and Meyer (1933). The right partner was simultaneously challenged with a pedicled full thickness skin graft from the left parabiont. The left parabiont received no skin graft and was killed after separation of the parabionts.

The skin incisions extended from the base of the ear to the hind leg. Precautions were taken not to injure blood vessels issuing from the axilla of the left partner. A single artery (accompanied by 2 veins) was found sufficient to nourish a pedicled graft 2–4 cm. in diameter. The ventral lips of skin incisions were united with a continuous suture. The abdominal walls were then incised and the 4 lips sutured together without coelio-anastomosis (common abdominal cavity). A skin flap was prepared from the dorsal lip of the skin wound in the left partner. The edges of the proximal half of the flap were stitched together to form a tubed pedicle; the distal free end was sutured into a skin defect on the back of the right partner (Fig. 1). The defect went down to muscular fascia and the corresponding graft was of full skin thickness including subcutaneous tissue to preserve blood vessels. The dorsal lips of skin wounds were then united with continuous sutures. A strong silk was placed through the scapulae of both partners. Ether anaesthesia combined with urethane (100

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mg./100 g. body-weight) was used. Postoperatively saline was given s.c. for several days to prevent thirst and consequent fighting.

The animals were separated 4, 5, 6, 7, 8, 9, 11 and 15 days following the operation. The tubed pedicle was cut as near the graft as possible and the grafts were inspected daily thereafter. Intermediate stages of destruction of the graft were estimated by gross inspection and the day of complete breakdown was taken to represent the time at which there was no surviving graft epithelium. Only those recipients that lived to be surgically separated on the predetermined day were taken into account and followed postoperatively.

RESULTS

The results are summarized in Table I. As can be seen, the survival time of pedicled grafts depends to a certain degree and with definite limitations on the duration of the parabiotic union. Out of 9 recipients separated on the 4th postoperative day only one displayed some degree of tolerance. The percentage of tolerant animals, *i.e.* of recipients that showed a survival time of grafts beyond 12 days, increases sharply in the groups of parabionts separated on the 5th and 6th postoperative day. The prolongation of parabiotic state to 7, 8 and 9 days does not result in any further increase of tolerant animals. No recipient separated on the 11th postoperative day succeeded in rejecting its graft by the 12th postoperative day. The graft seems to be protected by the tubed pedicle, but this protection is only temporary. Involution of the graft begins around the 10th postoperative day ; the graft becomes reduced in size and remains attached to the graft bed by a thin band of fibrous tissue. Most parabiotic pairs show partial dehiscence of the line of junction by the 11th postoperative day and spontaneous separation is complete with rare exceptions by the 15th postoperative day. In this series of experiments only one pair lived to be separated on the 15th postoperative day, and even in this pair partial dehiscence of the line of junction was present. Other pairs were lost either through spontaneous separation of partners or because of the death of one parabiont as a result of " parabiotic disease ". Attempts to maintain parabiotic union to the 20th postoperative day or beyond were unsuccessful in this strain combination.

TABLE I.—*Influence of Duration of Parabiotic Union on Survival Time of Pedicled Grafts*

Day of separation (postoperative)	Number of recipients	Graft destroyed by the 12th postoperative day	Graft alive beyond 12 days irrespective of fate of recipient
4	9	8/9	1/9 (11·1)*
5	15	9/15	6/15 (40)
6	12	2/12	10/12 (83·3)
7	7	2/7	5/7 (71·4)
8	4	1/4	3/4 (75)
9	6	2/6	4/6 (66·9)
11	7	0/7	7/7 (100)
15	1	0/1	1/1 (100)

* Figures in brackets indicate percentage of tolerant recipients.

The 37 recipients whose grafts survived beyond 12 days (Table I, row 4) are again presented in Table II as tolerant animals. These comprise hosts which destroyed their grafts between the 13th and the 30th postoperative days ; those in which rejection of graft extended over any period longer than 30 days and those that succumbed to " parabiotic disease " carrying fully or partly preserved grafts.

In all these cases the grafts remained healthy at the time when grafts in control animals would have shown an advanced degree of epithelial breakdown. The first sign of immune reaction in the graft, if any, was a scaling of superficial layers of epidermis. Sometimes a wedgelike necrosis took place instead, quite superficial at the margins of the graft and involving deeper layers around the remnant of the tubed pedicle. The crust would slough off in a few days and regeneration would take place exclusively from the graft. Immune reaction in the graft was absent or weak in the hosts that died of " parabiotic disease " before the 20th postoperative day. After a variable period of quiescence, unless the animal died of " parabiotic disease ", one or more necrotic patches would appear —patches which healed by scar formation leaving the graft more or less reduced in size. These immune crises in the graft frequently recurred until complete destruction took place.

As seen from Table II, no recipient with a viable graft survived the 30th postoperative day if weighing 160 g. or less. The only survivors tolerant beyond that period are found among recipients with body-weight above 160 g. Most of these showed mild symptoms of " parabiotic disease " consisting of diarrhoea, moderate anaemia or loss of weight. Two tolerant hosts weighing more than 160 g. died of " parabiotic disease ". The immune crises in the graft and the host were never observed simultaneously. One host is still alive, 2 yr. after the operation, carrying a graft which has gone through many immune crises and has been reduced to half its original size. It is worthy of note that the life-span of the host has not been shortened.

DISCUSSION

When 2 animals are joined in parabiotic union both partners are antigenically stimulated and the process of active immunization begins simultaneously on both sides. As soon as cross circulation is established around the 4th day of parabiotic union an enormous number of blood cells is exchanged between the partners. It can be assumed that the number of cells passing from one side to the other will approximately be the same. By this time the process of immunization is well advanced and the first antibody carrying cells are liberated into the blood stream. More of these cells are present in the circulation after the peak of intracellular antibody formation, about 5–7 days following first contact with antigen (Wissler, Fitch, La Via and Gunderson, 1957).

If the process of immunization proceeds *pari passu* in both partners the same number of actively immunized cells will be exchanged between the parabiotic partners. The antibodies formed by one parabiont are directed against and will damage body constituents of the homologous partner including its antibody forming tissues. The extent of damage will depend on the number of actively immunized cells the host had received from its partner and host's immunological reactivity to the given antigen.

At the moment of the separation of parabionts the cross-transfusion of humoral antibodies and actively immunized cells is discontinued. The result of competition

TABLE II.—*Tolerant Recipients. Fate of Grafts and Hosts**

Day of separation of parabionts	Grafts destroyed between the 13th and 30th postoperative day		Grafts destroyed after 30th day		Recipients that died of PBD with preserved grafts	
	a†	b‡	a	b	a	b
4	1/9	15 (140)	—	—	—	—
5	2/15	16 (170)	2/15	75 (180)	2/15	28 (160)
		20 (140)		2 yr. (190)	—	16 (150)
6	7/12	13 (180)	2/12	46 (200)	1/12	21 (150)
		17 (150)	—	58 (190)	—	—
		19 (210)	—	—	—	—
		16 (210)	—	—	—	—
		15 (220)	—	—	—	—
		25 (200)	—	—	—	—
		17 (200)	—	—	—	—
7	3/7	14 (150)	—	—	2/7	22 (140)
		15 (170)	—	—	—	18 (190)
		18 (160)	—	—	—	—
8	2/4	14 (180)	—	—	1/4	20 (160)
		15 (160)	—	—	—	—
9	2/6	15 (210)	1/6	31 (170)	1/6	21 (230)
		20 (180)	—	—	—	—
11	5/7	22 (160)	1/7	35 (180)	1/7	18 (160)
		13 (140)	—	—	—	—
		15 (140)	—	—	—	—
		15 (160)	—	—	—	—
		14 (180)	—	—	—	—
15	1/1	17 (210)	—	—	—	—

* Data refer to 37 recipients from Table I, Row 4.
† Tolerant hosts/total number of recipients.
‡ Survival time with body weight (g.) in brackets.

EXPLANATION OF PLATES

FIG. 1.—Albino rats in parabiotic union. Tubed pedicle and graft in place.

FIG. 2.—Typical appearance of rat suffering from " parabiotic disease ".

FIG. 3.—Spleen in " parabiotic disease". Disappearance of malpighian bodies; marked reduction of lymphoid elements. Note necrotic area around thrombosed artery in upper left corner. H. and E. ×60.

FIG. 4.—Lymph-node in " parabiotic disease "; reduction in size and number of lymph-follicles. H. and E. ×60.

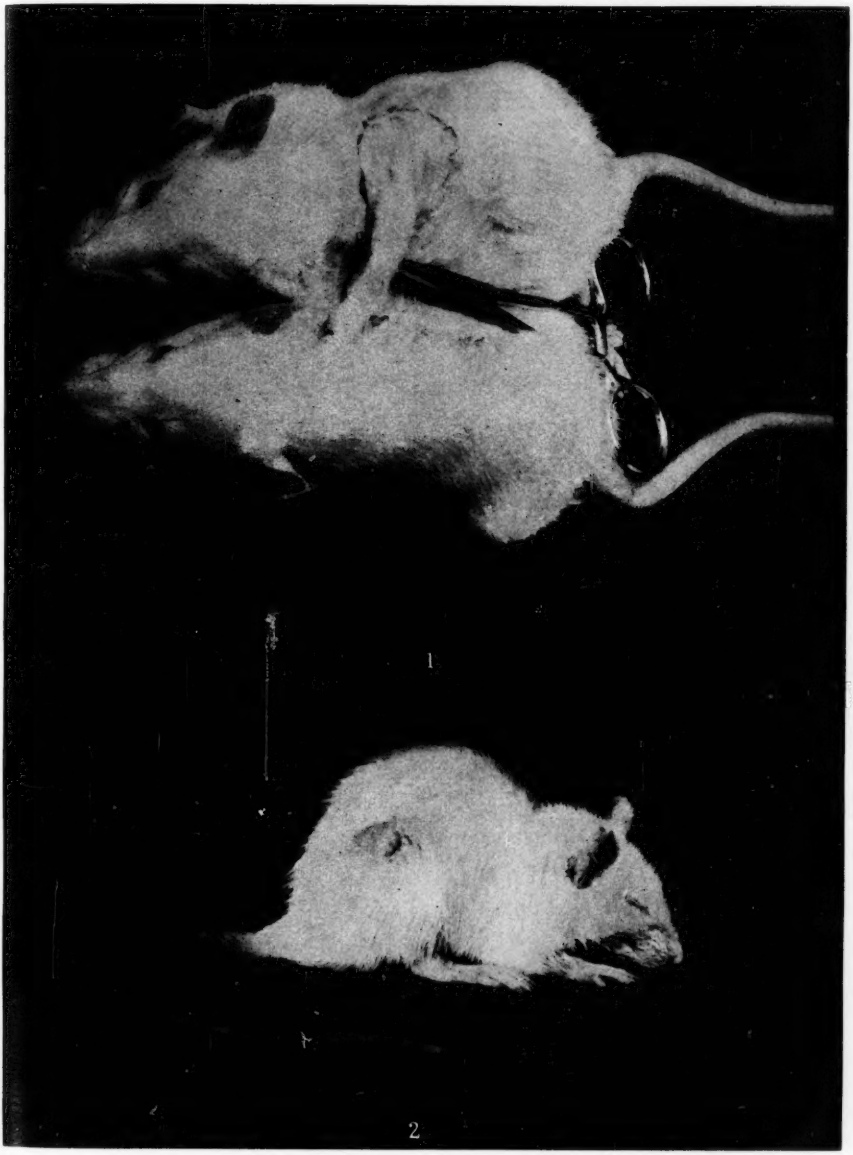
FIG. 5.—Thymus in " parabiotic disease "; reduced number of lymphoid elements. H. and E. ×150.

FIG. 6.—Marked hypoplasia of leukoblastic bone marrow in " parabiotic disease ". H. and E. ×240.

FIG. 7.—Liver in " parabiotic disease "; dense cellular infiltrations predominantly localized in periportal space and invading parenchyma of liver. H. and E. ×60.

FIG. 8.—Multiple necrotic foci in liver. H. and E. ×240.

FIG. 9 and 10.—Mucous membrane of small and large intestine showing necrosis and ulceration and covered with pseudo-membranous exudate. H. and E. ×150 and ×60 respectively.



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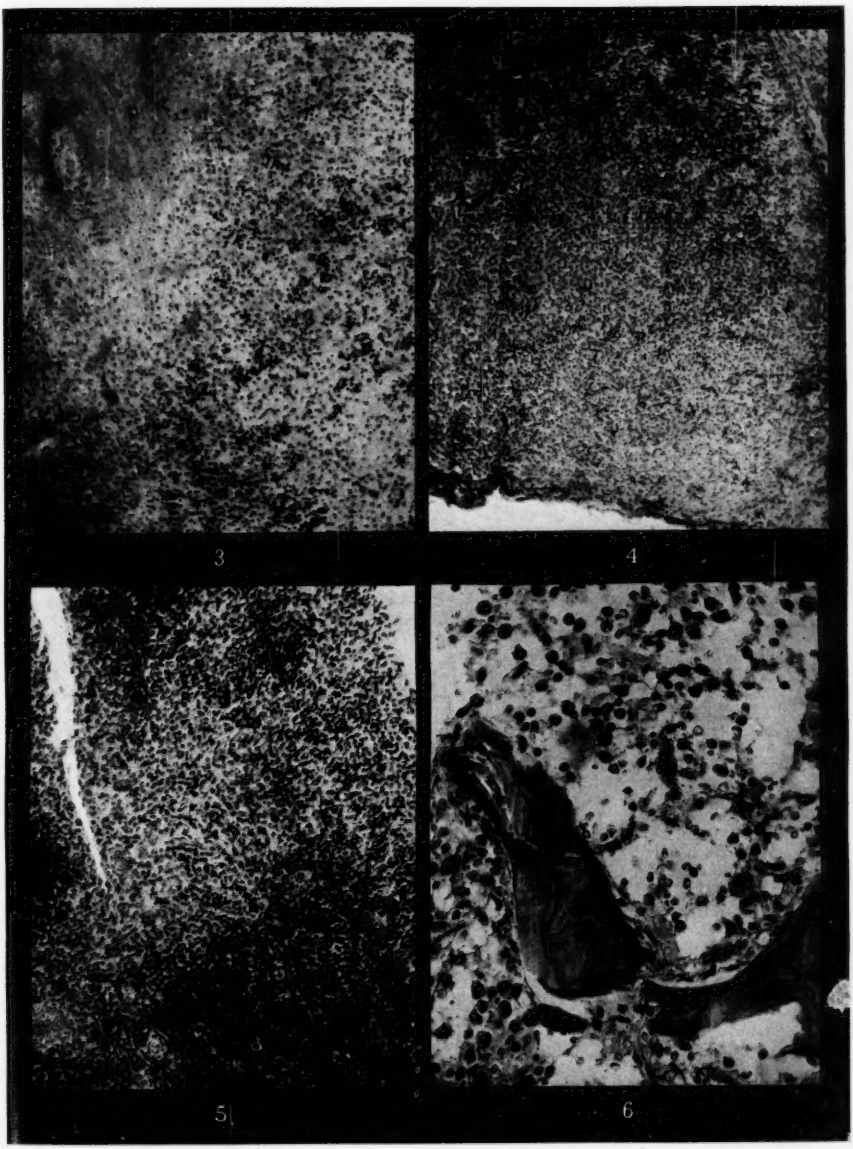

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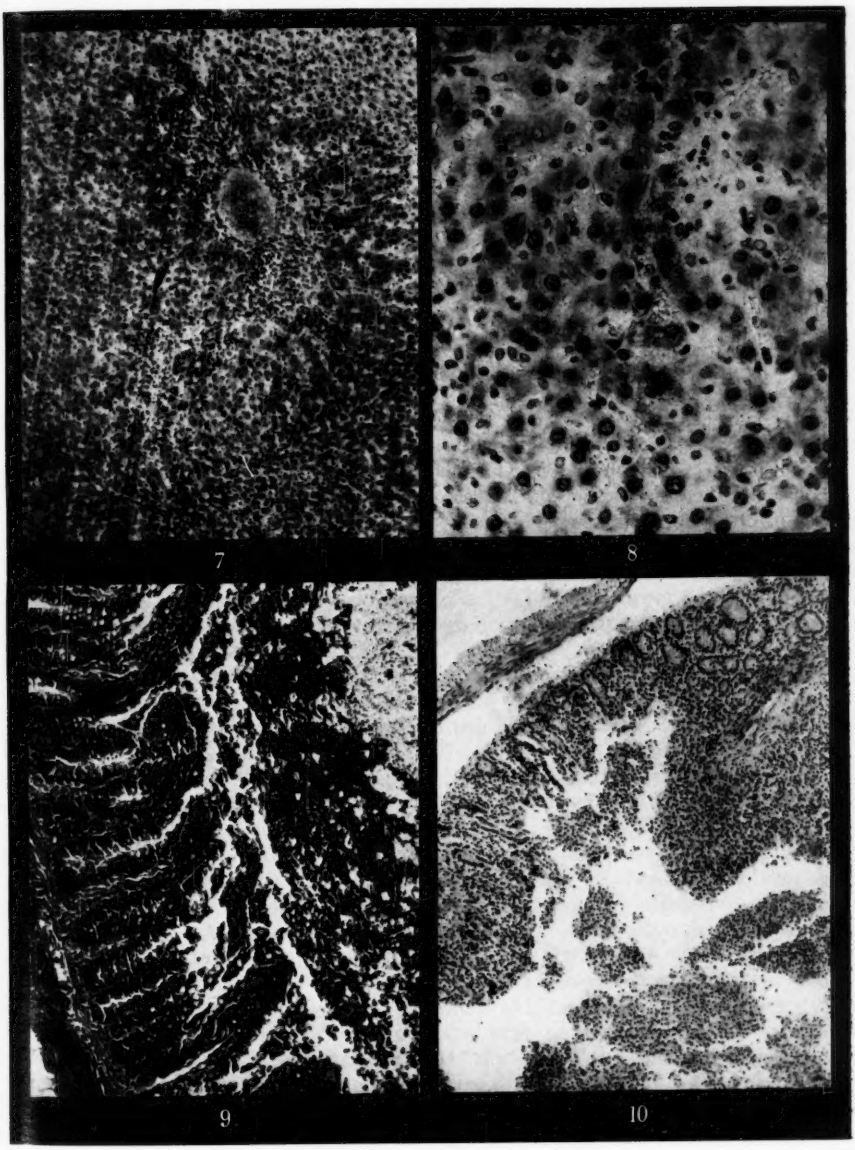

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between the host and donor's cells will be the inhibition of immune reaction as a consequence of damage to the lymphopoietic system of the host. This inhibition of immune reaction is primarily non-specific, as evidenced by the prolonged survival of skin grafts irrespective of their origin. Free skin grafts exchanged between homologous separated parabionts show prolonged survival time (Nakić *et al.*, 1960). The same is true if homologous parabionts are challenged with skin grafts from the 3rd strain ; that trauma was not responsible for this phenomenon was shown by joining in parabiotic union members of the same random-bred strain and challenging them with homologous grafts. Survival time of grafts was found to be within normal values (Nakić and Silobrčić, 1961).

If, however, the difference in immunological reactivity of the 2 partners is marked, the more slowly reacting partner will receive a much greater number of immunologically active cells than it will be capable of sending into its partner. Thus a vicious circle is set up. The greater the damage sustained by the lymphopoietic system of the more slowly reacting partner, the less will be its capacity to produce antibodies and destroy the donor's cells. That this damage to antibody producing tissues is not just a hypothetical possibility is shown by histological examination of animals suffering from " parabiotic disease " (Fig. 3, 4, 5, 6, 7, 8, 9, 10). Disappearance of lymphoid tissue from spleen, lymph-nodes and thymus and marked hypoplasia of leukoblastic bone marrow is a constant finding. As a result of this, the donor's cells are capable not only of surviving but also of proliferating in the host. From this moment the non-specific inhibition of immune reaction becomes specific " tolerance ". The immune system of the donor which has replaced that of the host will " tolerate " only donor-type grafts and will react against all tissue foreign to it. The first target is the host itself and " parabiotic disease " is a visible manifestation of attack by the donor's immune system against the host (Fig. 2).

The specificity of competitive tolerance is shown by the fact that the animal suffering from " parabiotic disease " is unable to destroy the skin graft from its partner but will reject the graft from the third strain (Nakić and Silobrčić, 1958 and 1961 ; Nakić *et al.*, 1960).

As shown in Table I, the incidence of tolerant animals is low in parabionts separated on the 4th postoperative day. This might be due to the relative inadequacy of the anastomotic channels to transport larger numbers of cells, but the more important reason is probably the small number of actively immunized cells in circulation. With the increase in the number of these cells in the blood stream in the following days, there is the increase in the incidence of tolerant recipients. The maximum is reached on the 6th postoperative day and nothing is gained if the duration of parabiotic union is extended to 7, 8 or 9 days. This is probably due to the fact that vascular channels connecting the 2 parabionts close from the 6th postoperative day on (Eichwald, Lustgraaf and Strainer, 1959). Although humoral antibodies can perhaps still pass through the parabiotic barrier, no more cells are exchanged once the functional separation has taken place despite the fact that partners may remain anatomically united for several days to come.

It is obvious from the foregoing that tolerance following parabiotic union cannot be of the all-or-nothing type either regarding duration or specificity. All degrees of inhibition of immune reaction, from slight prolongation of survival time of grafts to complete suppression of antibody formation by the host are

present. In the initial stages the inhibition of the immune reaction is non-specific and is a result of damage of the lymphopoietic system of one parabiont caused by actively immunized cells of the other. This damage can be bilateral and grafts irrespective of origin may show prolonged survival time on both partners. The recovery of the lymphopoietic system will depend on the interacting immunological forces of the 2 partners. Should one partner prevail, temporary or permanent replacement of the immune system of the more slowly reacting partner will ensue and specific tolerance will result.

This brings up the question of donor-host combination as a very important factor in inducing tolerance. If there is no significant difference in immunological reactivity toward a given antigen between the members of two strains neither lasting nor specific tolerance can be induced.

Replacement of the immune system might be a mechanism whereby specific tolerance is maintained in lethally X-irradiated animals treated with homologous bone marrow. This suggestion was already made by Trentin (1957*a*). The tolerant animals accept skin grafts from donors of bone marrow but will reject grafts of other origin (Trentin, 1957*b*) ; the same is true of heterografts (Zaalberg, Vos and van Bekkum, 1957). The host's lymphoid tissue may return and animals may in some cases contain a very small proportion of donor's cells but can still be grafted successfully with donor's skin (Barnes, Ford and Loutit, 1959). Indirect evidence that replacement of the immune system of the host by that of the donor may be only partial and yet compatible with tolerance comes from experiments described herein. A tolerant separated parabiont may recover from " parabiotic disease " with the graft soon showing signs of an immune crisis in the form of necrotic patches. If the crisis subsides and regeneration of the graft takes place the host may again show symptoms of " parabiotic disease ". These alternating crises may recur until the graft is destroyed or the host dies.

Whether the idea of competitive replacement could equally well be extended to the phenomenon of acquired tolerance in the sense of Billingham, Brent and Medawar (1953) remains to be seen. Assuming that the replacement of the immune system is the underlying mechanism one could explain for example why acquired tolerance of any major degree can be induced only with immunologically competent cells (Billingham and Brent, 1959) and why cell-free material is ineffective (Billingham, Brent and Medawar, 1958 ; Haškova, 1959). Perhaps the most convincing proof in favour of replacement is the low incidence and degree of tolerance induced in new-born mice of parental strain with adult F_1 hybrid spleen cells (Billingham and Brent, 1959). Woodruff and Michie (personal communication) also attempted to induce tolerance by i.v. injection of 3–20 $\times 10^6$ spleen cells from (C57 $\times$ CBA) F_1 mice into neonatal recipients of the parent strains. They produced negligible tolerance in the C57 recipients and only slight tolerance in the CBA recipients. In subsequent experiments they found that irradiating recipients (200 r) prior to the injection made no difference.

If this be so, specific tolerance to living tissue, whether homologous or heterologous, and irrespective of the way it is induced, would not be a deviation from the normal immunological response mechanism but an expression of it.

Medawar (personal communication) admits the possibility that competitive replacement might account for some fraction of acquired tolerance under certain circumstances, but he feels that we should not propose it without taking into account the following facts : (*a*) embryonic cells may induce tolerance with no

trace of " runt disease " presumably because they become tolerant of the host antigens ; the tolerant animals so formed are stable chimaeras (Billingham and Brent, 1959) ; (*b*) in comparatively " easy " systems adult hybrid cells will induce tolerance quite effectively when injected into parental animals, though they cannot be immunologically competent in this situation (CBA/A → A) ; (*c*) adult cells which are not immunologically reactive can induce tolerance, *e.g.* pure blood monocytes in birds (Terasaki, 1959) ; (*d*) in " easy " systems, tolerance can be induced by cell-free extracts, *e.g.* the male to female system (Billingham and Silvers, 1960).

Medawar, therefore, suggests that in the light of these facts we should perhaps say that some fraction of tolerance may be attributable to competitive replacement when tolerance is in fact induced by the use of adult lymphoid cells.

The fact that foetal cells are capable of inducing tolerance without causing symptoms of " runt disease " might indeed constitute a strong argument in favour of acquired tolerance. However, another explanation is at hand if one remembers that the susceptibility of hosts to the " graft-versus-host " reaction declines as their age at the time of inoculation increases (Cock and Simonsen, 1958 ; Billingham and Brent, 1959). A similar phenomenon observed in rats is reported in this communication. As Billingham and Brent have shown (1959) injection of adult parental spleen cells into F_1 hybrids 23 days old is completely ineffective as far as runting is concerned ; even 12 days old recipients are well resistant when compared with neonatal animals which all die of acute " runt disease ". Now, when tolerance is conferred naturally as the result of anastomosis between the foetal blood circulations (Anderson, Billingham, Lampkin and Medawar, 1951) or, artificially, by the parabiosis of chick embryos or by the intravenous injection of chick embryos with homologous embryonic blood (Hašek and Hraba, 1955 ; Billingham, Brent and Medawar, 1956 ; Simonsen, 1957) 2 sets of cells are brought together both immunologically inactive until some time after birth. When the process of active immunization begins, the sequence of events will probably be much the same as in adult parabionts ; the interaction of competitive forces might bring about the non-specific inhibition of immune reaction as a result of damage to both immune systems. Further course will depend on the immunological reactivity of the parties concerned, but whatever the final outcome, the recipient by this time will be well out of danger period.

The competitive replacement tolerance excludes *per se* conditions such as " immunological unresponsiveness " resulting from injection of a variety of heterologous proteins into neonatal or adult rabbits (Weigle and Dixon, 1959) or " immunological paralysis " induced in mice with large doses of pneumococcal polysaccharide (Felton, 1949 ; Stark, 1959). Suppression of agglutinin formation following injection of homologous or heterologous red cells into unborn or newly-born birds (Simonsen, 1955 ; Billingham *et al.*, 1955 ; Hašek, 1956) is probably a similar phenomenon. Medawar and his colleagues (1959) failed to produce skin transplantation immunity or tolerance with purified red cells. However, one cannot totally disregard the above phenomena as possible contributory factors in the induction of tolerance to living tissues. In the so-called " difficult " systems, where immunological disparity between donor and recipient is very great the part played by this component may be negligible but in " easy " systems even a feeble degree of inhibition of immune reaction would suffice to prolong survival of transplanted cells.

We accept the contention that blood monocytes as such are incapable of producing antibody. They might be able, however, to give rise, through division and differentiation, to immunologically competent cells. There is some evidence that blood monocytes could be the totipotential primitive mesenchymal cells (Bond, Cronkite, Fliedner and Schork, 1958).

The attempt to induce tolerance by cell-free extracts in "easy" systems, such as the male to female system has had a very limited success (Billingham and Silvers, 1960). Repeated inoculations of baby mice with relatively high dosages of cell-free antigenic preparations induced tolerance of the Y factor in about 20 per cent of the subjects. The authors consider the results as disappointing in view of the high degree of tolerance-responsiveness of baby C57 females to intact male cells.

SUMMARY

Tolerance to skin homografts was induced in adult albino rats from two genetically unrelated strains by uniting them in parabiosis while attaching a pedicled skin graft from one to the other.

Tolerance to homografts in adult animals is not necessarily connected with fatal manifestations of a graft-versus-host reaction. The incidence of tolerant animals depends with definite limitations on duration of parabiotic union.

The replacement of the immune system of the host by that of the donor is discussed as a possible mechanism responsible for induction of tolerance in parabionts.

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CHANGES IN THE ACTIVITY OF THE RETICULO-ENDOTHELIAL SYSTEM (RES) FOLLOWING THE INJECTION OF PARENTAL SPLEEN CELLS INTO F_1 HYBRID MICE

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THE transplantation to F_1 hybrid mice of splenic or lymphoid cells from immunologically mature members of either parental strain leads to the development of a graft-versus-host reaction, as the recipient is genetically tolerant of the donor cells. Some of the features of the reaction developing in this situation have been described by Schwartz, Upton and Congdon (1957); Cole and Ellis (1958); Trentin (1958, 1959); Billingham and Brent (1959) and Gorer and Boyse (1959). In summary, it appears that resistance to the lethal effect of graft-versus-host reaction in F_1 hybrids increases with age, but is greatly diminished by sub-lethal irradiation. Isoimmune parental donor cells are more effective than non-immune cells, whilst a negative correlation exists between the dose of cells injected and the survival time of the hybrid recipients.

The mortality rates obtained have varied greatly among the different inbred strains investigated, but in some cases additional evidence for graft-versus-host reaction has been inferred from loss in body-weight in surviving animals (Trentin, 1958; Billingham and Brent, 1959). Simonsen, Engelbreth-Holm, Jensen and Poulsen (1958) found that enlargement of the spleen and, less markedly, the liver was a more reliable index of graft-versus-host reaction in F_1 hybrids than reduction in body-weight. Gorer and Boyse (1959) described the dominant proliferating cell type in the spleen and liver of F_1 hybrids undergoing the reaction as a histiocyte. It seemed possible, therefore, that proliferation of reticulo-endothelial (RE) elements might be detected by an increase in phagocytic function, thus providing a convenient *in vivo* graft-versus-host assay.

MATERIALS AND METHODS

Mice.—Adult animals were used belonging to the inbred strains CBA, C57BL and A maintained in this Department, and F_1 hybrids derived from them. The CBA and C57BL colonies are sub-lines of those maintained at the MRC Radiobiological Research Unit, Harwell, and the A colony is a sub-line of that maintained by Dr. N. A. Mitchison, Department of Zoology, Edinburgh University.

Spleen cell suspensions.—Spleens were removed aseptically from freshly killed mice and broken up gently in pH 7·4 Tris-buffered Ringer solution, by means of a loose-fitting ground glass homogeniser. A volume of approximately 2 ml. per spleen was used. The resultant coarse suspension was filtered through a fine-meshed stainless steel sieve and centrifuged at 800 rpm for 7 min. The supernatant was discarded and the cells resuspended in fresh Ringer by repeatedly sucking in and out of a Pasteur pipette. Finally, the suspension was allowed to stand for 4 min. before use, enabling large particles to settle out, as recommended by Billingham and Brent (1959). The cell suspension was not tolerated by the intravenous route if this last step was omitted. The concentration of nucleated cells used did not exceed 500,000 per c.mm. Intravenous injections were made into a tail vein with a volume not

exceeding 0·3 ml. Viable counts, which were performed from time to time on the suspensions by the trypan blue method, were never lower than 85 per cent.

Iso-immunization.—One parental strain was immunized against the other by allowing it to reject an orthotopic skin homograft. Full thickness skin grafts were made by the method of Billingham and Medawar (1951). The recipient was killed and its spleen removed 14 days after grafting.

Quantitative estimation of the phagocytic activity of the reticulo-endothelial system (RES).—The rate of disappearance from the blood of a standard dose of stable colloidal carbon (16 mg./100 g. body weight, preparation C 11/1431a) was measured as described by Biozzi, Benacerraf, Stiffel and Halpern (1954). After the carbon was injected intravenously into a tail vein, repeated blood samples were removed from the retro-orbital venous plexus, lysed in 0·1 per cent Na_2CO_3 and the amount of carbon estimated in a photo-electric absorptiometer. The blood clearance of carbon follows an exponential function, so that the phagocytic index " K " =

$$\frac{\text{log. concentration } a - \text{log. concentration } b}{tb - ta}$$

where ta and tb = time in min. at a and b.

X-irradiation.—Whole-body X-irradiation was given as a vertical beam from a Westinghouse machine under the following conditions: 230 kV at 15 ma; target-skin distance 50 cm.; 0·5 mm. Cu and 1·0 mm. Al filters; dose rate in air 142 r per min. The mice were placed in a rectangular perforated polystyrene box (13·5 × 7·5 × 3·2 cm.).

Histological techniques. (*a*) Silver impregnation.—Tissues were fixed in neutral formalin, sections cut 15–20 μ thick and stained by Marshall's (1956) modification of the Weil-Davenport method. (*b*) Pyronin-methyl green staining.—Several different staining schedules were tried. The best results were obtained by fixation in alcoholic formalin followed by staining of sections 7 μ thick by the modification of Carleton and Leach (1938).

Nomenclature of mesenchymal cell types.—Cells in the liver and spleen were classified as reticulo-endothelial cells only if they phagocytosed colloidal carbon *in vivo* or were argyrophilic in tissue sections. Cells were designated primitive reticular cell, activated reticulum cell, plasmablast and immature and mature plasma cell, in pyronin-methyl green sections, according to the descriptions of Marshall and White (1950).

RESULTS

The phagocytic indices determined on individual F_1 (C57BL × CBA) mice at various intervals after the injection of parental or isologous spleen cells are shown in Table I. At no time did any value obtained in hybrids injected with isologous cells (*i.e.* cells from genetically identical hybrids) diverge from the normal range (K = 0·010–0·020). Following the injection of C57BL spleen cells, progressively higher phagocytic indices were found during the second week, which reached a high maximum at 15 days and fell steeply by 20 days. A smaller and less uniform but essentially similar response followed the injection of CBA spleen cells. In view of the apparent variation in the extent of RE stimulation at any one time, it seemed preferable to perform repeated " K " estimations on the same mice and obtain individual response curves rather than to rely on one reading. This method proved satisfactory, for the test dose of carbon used (16 mg./100 g.) did not itself stimulate the RES so as to affect subsequent " K " estimations in the isologous cell-injected controls. It was technically possible to make 3–4 estimations during the required period, but removal of further blood samples seemed undesirable in view of the anaemia usually accompanying graft versus host reaction in these mice. The pattern of RE stimulation in individual F_1 (C57BL × CBA) mice following injection of parental spleen cells is shown in Fig. 1, where the much greater effect following the injection of C57BL cells rather than CBA

cells is clearly seen. Disproportion in the extent of stimulation of the phagocytic index produced by the 2 parental strain spleen cells was even more pronounced following their injection into F_1 (CBA × A) hybrids (Fig. 2). In this case, the maximum value for " K " in mice injected with A cells was increased 7–8-fold, whereas in mice injected with CBA cells the rise did not exceed 2 times normal.

The assumption that stimulation of the RES was caused by an immunological reaction of the injected spleen cells against the hybrid host was confirmed by the

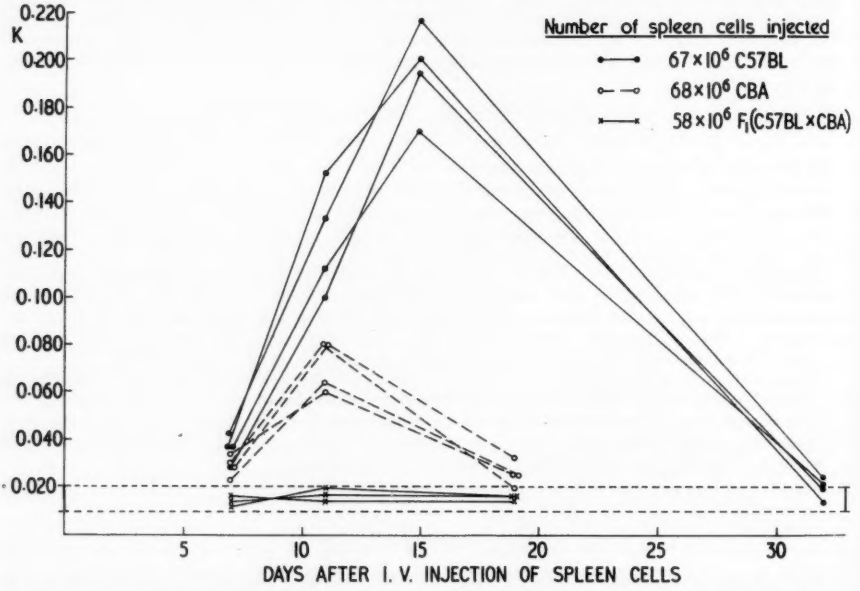


FIG. 1.—Changes in the phagocytic index " K" following the injection of parental or isologous spleen cells into F_1 (C57BL × CBA) mice. The lines joining consecutive points represent individual animals. The dotted horizontal lines indicate the limits for normal values.

TABLE I.—*The Mean Phagocytic Indices in F_1 (C57BL × CBA) Mice at Varying Times after the Intravenous Injection of Parental or Isologous Spleen Cells*

Days after I.V. injection of spleen cells	Type of cells injected		
	C57BL	CBA	F_1 (C57BL × CBA)
5	0·021 (3)*	—	0·018 (3)
7	0·018 (3)	0·021 (4)	0·015 (3)
9	—	0·024 (3)	—
11	0·056 (3)	0·034 (4)	0·014 (3)
13	0·109 (3)	0·020 (3)	0·013 (3)
14	0·116 (3)	—	—
15	0·140 (11)	0·084 (8)	0·014 (3)
20	0·039 (3)	0·017 (3)	0·012 (3)

* The number of animals represented in each determination is expressed in brackets. The dose of spleen cells used was 45–95 × 10^6. Female mice were used as donors and recipients in this and subsequent experiments, except where otherwise stated.

use of isoimmune cells (*viz.* spleen cells from one parental strain which had been previously immunized against the other). The results in Fig. 3 show that when isoimmune C57BL or CBA spleen cells were injected into F_1 (C57BL × CBA) mice, RE stimulation was advanced by at least 3 days as judged by the readings at day 5 and the time of the peak reaction (Table I and Fig. 1). Whereas the reaction produced by isoimmune C57BL cells attained a uniformly lower peak value than that produced by normal cells, isoimmune CBA cells led to a higher peak than normal cells in 2 out of 5 mice.

The phagocytic index usually returned to a value at or only slightly above normal within 4–5 weeks of the cell injection. However, in some animals slightly higher values (K = 0·030–0·050) tended to persist for very much longer.

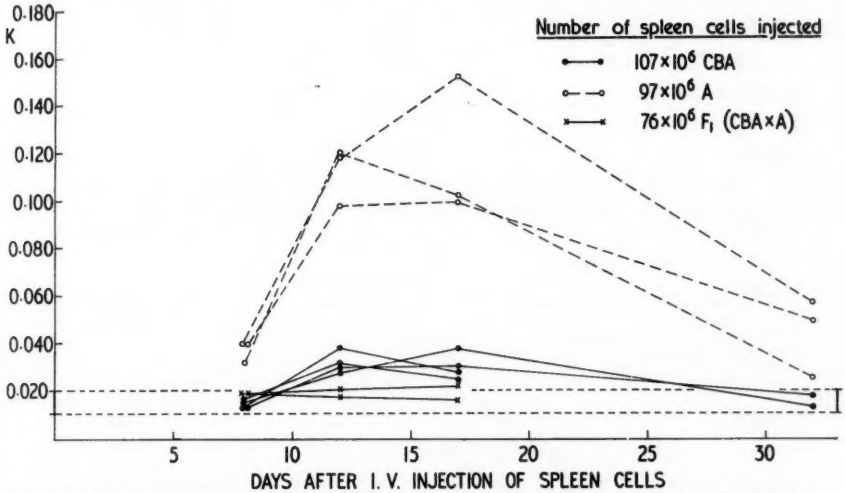


FIG. 2.—Changes in the phagocytic index " K " following the injection of parental or isologous spleen cells into F_1 (CBA × A) mice. As Fig. 1.

In view of the fact that young adult hybrids are known to be more sensitive to graft-versus-host reaction than older animals, the effect on the phagocytic index of injecting C57BL spleen cells into F_1 (C57BL × CBA) mice of different age groups was investigated (Table II). The results show that " K " in young adults reached a value twice that produced in old adults when a large dose of cells was injected. On the other hand, a small dose (16×10^6/20 g.) produced no significant rise in " K " in either age group. (As a dose of 40×10^6 had been shown to increase the phagocytic index, the minimal effective dose lies somewhere between 16×10^6 and 40×10^6.)

The effect of previous irradiation of the recipient on the RE reaction following the injection of parental spleen cells was investigated for two reasons. First, sub-lethal irradiation is known greatly to increase the mortality rate from graft-versus-host reactions in F_1 hybrids (*e.g.* Table IV). Secondly, LD_{50} irradiation in rats has been shown to prevent the usual proliferative response of the RES which follows the injection of zymosan (Benacerraf, Kivy-Rosenberg, Sebestyen and

Zweifach, 1959). However, it appeared that both sub-lethal (360 r) and lethal (850 r) irradiation were unable to prevent the hyperphagocytic response of the RES which follows the injection of C57BL spleen cells into F_1 (C57BL × CBA)

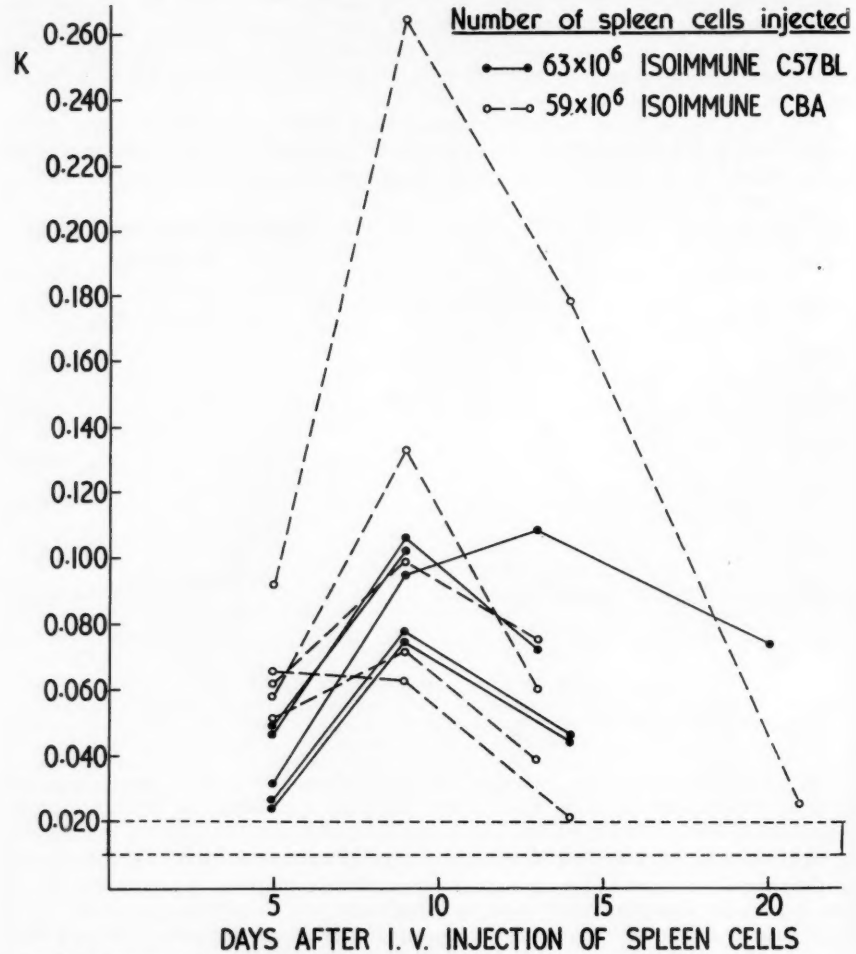


FIG. 3.—Changes in the phagocytic index " K " following the injection of isoimmune parental spleen cells into F_1 (C57BL × CBA) mice. As Fig. 1.

mice (Table III). The significance of this finding will be considered in the discussion.

A smaller and earlier rise in phagocytic index may also accompany the rejection of hybrid spleen cells in the parental strain by the host-versus-graft reaction. For example, a dose of 74×10^6 F_1 (C57BL × CBA) female spleen

TABLE II.—*The Relation of the Number of C57BL Spleen Cells Injected to the Extent of RES Stimulation in Old and Young Adult F_1 (C57BL × CBA) Mice*

Dose of C57BL spleen cells I.V. (per 20 g.)	Phagocytic index "K"* Weight of mice: 14–15 g. Individual readings	14–15 g. Mean	29–31 g. Individual readings	29–31 g. Mean
105 × 10^6	0·176 0·162 0·122	0·153	0·103 0·056 0·054	0·073
16 × 10^6	0·022 0·021 0·019	0·021	0·021 0·019 0·016	0·019

* The phagocytic indices were determined 14 days after the injection of cells.

TABLE III.—*The Failure of Previous Whole-body Irradiation to F_1 (C57BL × CBA) Mice to Prevent Stimulation of the RES following the Injection of C57BL Spleen Cells*

Irradiation dosage	Dose of C57BL spleen cells injected I.V.	Phagocytic index "K": Irradiated mice		Unirradiated mice	
		14 days after injection of spleen cells			
360 r 48 hr. before injection of spleen cells	49 × 10^6 cells	0·150 0·147 0·106 0·088 0·076	0·113 (mean)	0·166 0·108 0·073	0·116 (mean)
		8 days after injection of spleen cells			
850 r 3 hr. before injection of spleen cells	98 × 10^6 cells	0·072 0·058 0·051 0·041	0·056 (mean)	0·068 0·060 0·054	0·061 (mean)

Male mice were used as recipients and females as donors in this experiment.

TABLE IV.—*The Mortality Rates and Survival Times of F_1 (C57BL × CBA) Mice following the Intravenous Injection of Parental Spleen Cells*

Spleen cells injected	Dose (× 10^6)	Number of deaths / Number of injected mice	Survival times of animals dying (days)
C57BL	75–110	5/14*	19, 19, 35, 35, 40
CBA	75	1/15*	27
C57BL into irradiated (360 r) recipients	50	6/6	19, 19, 22, 24, 26, 26

* A similar difference in mortality rate between mice injected with C57BL and CBA cells has been found consistently in other experiments.

cells was injected into 3 animals of each parental line. Three days later, the mean " K " in the C57BL group was 0·054, falling to 0·028 at day 7 and 0·018 at day 12. On the other hand, the mean " K " at day 3 in the CBA group was only 0·021, falling to 0·016 at day 8.

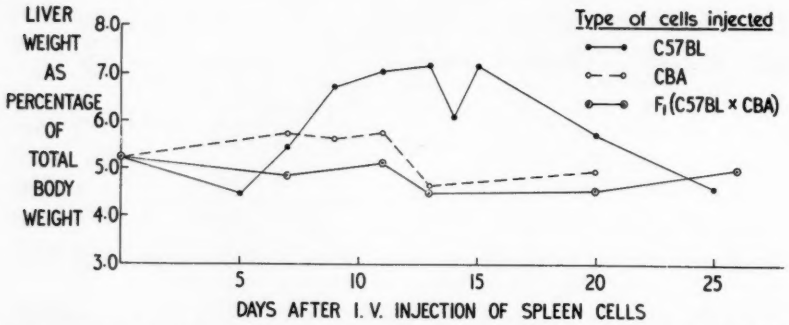


FIG. 4.—Changes in the weight of the liver following the injection of parental or isologous spleen cells into F_1 (C57BL × CBA) mice. Each point represents the mean value for 3 animals. The dose of spleen cells used was 45–95 × 10^6.

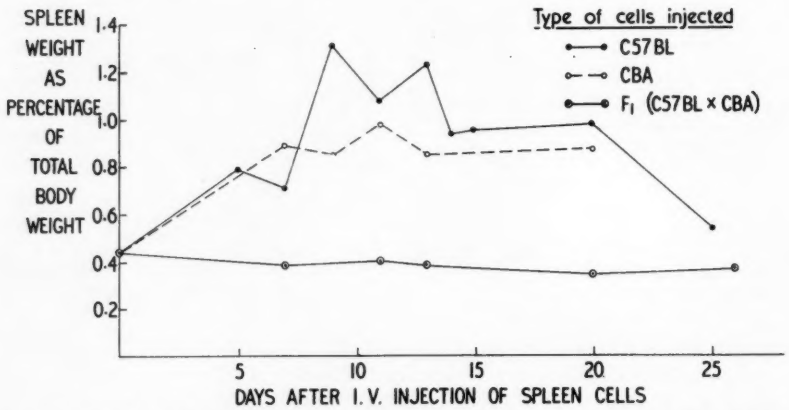


FIG. 5.—Changes in the weight of the spleen following the injection of parental or isologous spleen cells into F_1 (C57BL × CBA) mice. As in Fig. 4.

Changes in the weight of the liver and spleen

Considerable enlargement of the liver accompanied the duration of phagocytic stimulation in F_1 (C57BL × CBA) mice injected with C57BL spleen cells (Fig. 4). On the other hand, very slight enlargement followed the injection of CBA spleen cells in parallel with a lesser effect on the RES. Both C57BL and CBA spleen cells produced a 2–3-fold enlargement of the spleen, the former having the slightly greater effect (Fig. 5). It is noteworthy that spleen enlargment precedes liver

enlargement by several days, at least following injection of C57BL cells. The size of the spleen subsequent to the period shown in Fig. 5 varied greatly : in moribund animals the spleen was usually shrunken and acellular, whereas it remained enlarged in some survivors 50–70 days after the original injection of spleen cells (in parallel with a " K " value higher than normal).

The greater rise in " K " produced by C57BL spleen cells as compared with CBA cells in F_1 (C57BL × CBA) mice correlates with a more severe graft-versus-host reaction as measured by increase in spleen and liver weights, loss in body weight (unpublished data) and mortality rate (Table IV). It was not possible, however, to predict from estimation of " K " which animals were doomed and which would survive. Furthermore, although 360 r irradiation of the recipients before injection of spleen cells produced a uniformly lethal reaction (Table IV), their values of " K " did not differ from comparable unirradiated recipients (Table III).

Histological changes

The enlarged liver showed a pronounced increase in the number of RE cells lining the sinusoids (Figs. 6 and 7), and many of these phagocytes were larger than normal. Conversely, the flattened endothelial cells, showing only slight accumulation of carbon granules, were greatly reduced in number. These changes, which could not be detected earlier than one week after the injection of spleen cells, reflected the extent of RE stimulation measured *in vivo*. Foci of proliferating cells, mainly non-phagocytic, were present around many of the portal tracts (Fig. 8). At the height of the reaction these consisted of primitive reticular cells and pyroninophilic types (plasmablasts and immature plasma cells), while 2–3 weeks later there was a shift towards the mature plasma cell type. The cells in such foci showed no tendency to invade the sinusoids, except in sub-lethally irradiated recipients (Fig. 9). In this case, cellular proliferation invariably led to distension of the sinusoids with consequent atrophy of liver parenchyma.

No increase in number of RE cells could be detected in the spleen by either carbon uptake or silver impregnation methods—in fact, the amount of carbon phagocytosed was much less than in control animals (Figs. 10 and 11). The dominant change in the spleen was an overwhelming proliferation of plasmablasts and immature plasma cells, which accounted for at least 50 per cent of the total cell population in the enlarged organ (Fig. 12). Large numbers of primitive reticular and activated reticulum cells were also present in the earlier stage of the reaction. Destruction of Malpighian follicles in the spleen was complete within 7–9 days of the injection of donor cells. Mitotic figures and multinucleate forms were common amongst the pyroninophilic cells. Regression of the reaction was accompanied by the presence of many mature plasma cells in the spleen (Fig. 13) and the regeneration of lymphoid follicles.

Several axillary lymph nodes examined showed a basically similar pathology to that described for the spleen.

DISCUSSION

Three criteria have been used previously for detecting graft-versus-host reactions in F_1 hybrid mice, namely mortality, loss in body weight and spleen enlargement. Other investigations mentioned earlier, as well as the present one,

have shown death to be unreliable as an indication of the reaction. Neither does body weight change seem to be satisfactory, for this was negligible in F_1 (C57BL $\times$ CBA) and F_1 (CBA $\times$ A) mice injected with CBA cells, whereas in both instances a significant rise in " K " was recorded. Estimation of the phagocytic activity of the RES clearly provides a useful index of graft-versus-host reaction in F_1 hybrids, probably of the same order of sensitivity as enlargement of the spleen; but it possesses the added advantage that the reaction can be detected *in vivo* and the animal subsequently used for further immunological investigation. Whether or not this technique can be applied to other situations allowing graft-versus-host reaction—radiation chimaeras and mice injected with homologous lymphoid cells at birth—remains to be determined.

The proliferating cells in both spleen and liver of F_1 (C57BL $\times$ A) mice injected with parental spleen cells were described by Gorer and Boyse (1959) as histiocytes. The results recorded in the present work, using F_1 (C57BL $\times$ CBA) and F_1 (CBA $\times$ A) hybrids, disagree with this interpretation in that the proliferating cells in the spleen and periportal tracts of the liver were mainly pyroninophilic (plasmablasts and immature plasma cells) and not RE cells. An increase in number of the latter type was found solely in the liver sinusoids.

One unsettled point is whether the raised phagocytic index is effected by an increase in the number of RE cells of donor or host origin or both. For the present, the most likely interpretation seems to be that it represents a response of RE cells to the continuous accumulation of dead cells in foci of graft-versus-host reaction. Proliferation of pyroninophilic cells and disappearance of Malpighian follicles in the spleen precedes the RE response by several days. That the RE cells concerned are of host origin is suggested by the histology of the stimulated hepatic RE cells, which is essentially similar to that produced by injection of bacterial endotoxin and colloids where obviously only host cells are concerned (Howard,

EXPLANATION OF PLATES

FIG. 6.—Liver of F_1 (C57BL $\times$ CBA) mouse 15 days after injection of isologous spleen cells. Colloidal carbon was injected 6 hr. before removal of liver. The normal distribution of carbon-containing RE cells is shown. Basic fuchsin. $\times 85$.

FIG. 7.—Liver of F_1 (C57BL $\times$ CBA) mouse 15 days after injection of C57BL spleen cells, showing great increase in number of carbon-containing RE cells. As Fig. 6. Basic fuchsin. $\times$ 85.

FIG. 8.—Liver of F_1 (C57BL $\times$ CBA) mouse 10 days after injection of C57BL spleen cells. Colloidal carbon was injected 6 hr. before removal of liver. Note proliferation of non-phagocytic cells around portal tract. Pyronin-methyl green. $\times$ 170.

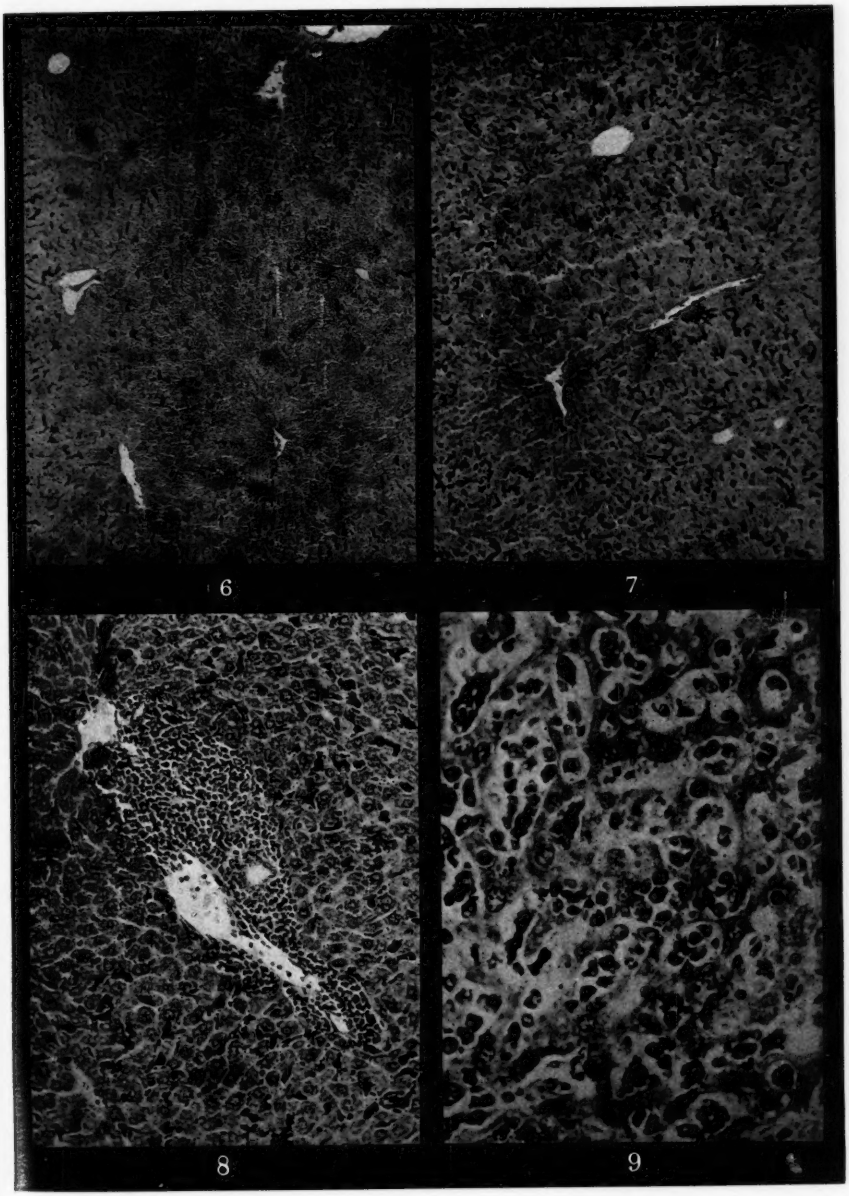
FIG. 9.—Liver of 360 r irradiated F_1 (C57BL $\times$ CBA) mouse 24 days after injection of C57BL spleen cells. Colloidal carbon was injected 6 hr. before the removal of liver. Note presence of proliferating non-phagocytic cells as well as phagocytes in the dilated sinusoids. Pyronin-methyl green. $\times 340$.

FIG. 10.—Spleen of mouse shown in Fig. 6. The normal distribution of carbon-containing RE cells in the pulp is shown. Basic fuchsin. $\times 85$.

FIG. 11.—Spleen of mouse shown in Fig. 7. Note the great reduction in uptake of carbon, as compared with Fig. 10. Basic fuchsin. $\times 85$.

FIG. 12.—Spleen of F_1 (C57BL $\times$ CBA) mouse 15 days after injection of C57BL spleen cells. Immature plasma cells, plasmablasts and pyroninophilic cells undergoing mitosis are shown. Pyronin-methyl green. $\times 680$.

FIG. 13.—Spleen of F_1 (C57BL $\times$ CBA) mouse 54 days after injection of CBA spleen cells. Note the presence of many mature plasma cells. Pyronin-methyl green. $\times 680$.



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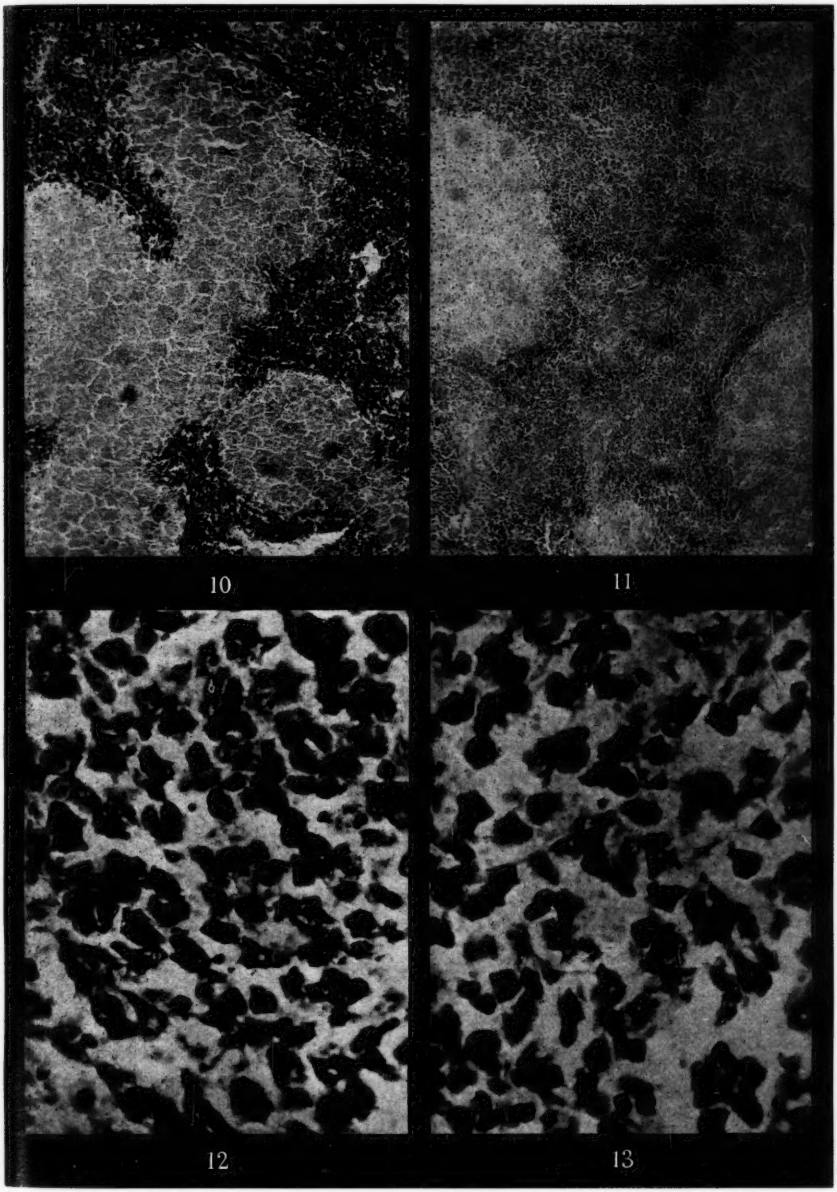

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Rowley and Wardlaw, 1958; Howard, 1959). Furthermore, the increase in number of RE cells in the liver was accompanied by a decrease in the number of hepatic endothelial cells as in endotoxin-stimulated mice, where a transformation of endothelial cells into RE cells has been proposed (Howard, 1959). The fact that the hyperphagocytic response could be elicited equally well in lethally irradiated recipients of parental spleen cells suggested initially that donor cells might be responsible. This interpretation was subsequently invalidated by the finding that endotoxin could stimulate the RES in similarly irradiated mice (Howard, unpublished data).

A second problem for further investigation is to elucidate the significance of the return to normal phagocytic activity in surviving animals. This may be explained by death of the donor cells (Boyse, 1959) or by survival of donor cells rendered specifically tolerant of host antigens (Simonsen, 1960) or possibly by donor cells becoming immunologically inert. The fate of the donor cells may vary according to the strain combinations used.

SUMMARY

Graft-versus-host reaction was induced in adult F_1 hybrid mice (C57BL × CBA and CBA × A) by the intravenous injection of parental strain spleen cells and the phagocytic activity of the RES assessed at intervals thereafter by following the rate of elimination of colloidal carbon from the blood. After 7 days a rise in RE activity commenced which reached a maximum (up to 10 times normal) at 15 days and returned to a more or less normal level by 20–40 days. Similar stimulation could be obtained in sub-lethally or lethally irradiated recipients. The immune basis for the reaction was confirmed by the finding that isoimmune spleen cells provoked stimulation 3–4 days earlier than normal cells. Spleen cells from one parental line consistently produced a greater degree of RE stimulation than the other in both hybrid strains, in parallel with the severity of the reaction assessed by other criteria.

A great increase in the number of active RE cells was found in the liver during the reaction. The normal structure of the spleen was destroyed and the organ filled with proliferating pyroninophilic cells, identified mainly as plasmablasts and immature plasma cells. Similar cells accumulated in the periportal tracts of the liver.

It is considered that the carbon clearance test provides a sensitive method for detecting the presence of graft-versus-host reaction *in vivo*.

I am indebted to Professor M. F. A. Woodruff for his interest in this work.

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ANALYSIS OF THE HOST-PARASITE EQUILIBRIUM IN CHRONIC MURINE TUBERCULOSIS BY TOTAL AND VIABLE BACILLARY COUNTS

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TUBERCULOSIS both in man and in experimental animals can manifest itself in almost any degree of severity and progression, from a relatively acute and progressive generalized infection to one in which the infection remains localized and apparently stable. The latter type of disease is a manifestation of partial immunity, in which not all the tubercle bacilli are destroyed and in which the surviving bacilli remain in equilibrium with the host, often for long periods. Analysis of the resulting host-parasite equilibrium is pertinent to our understanding of the type of immune process frequently seen in tuberculosis.

In mice chronic tuberculosis can be readily produced as a predominantly pulmonary infection either by giving small doses of tubercle bacilli (Hart and Rees, 1950 and 1960 ; McCune and Tompsett, 1956 and McCune, Tompsett and McDermott, 1956) or by challenging with larger doses of virulent tubercle bacilli following vaccination with BCG (Sever and Youmans, 1957). Under both conditions of infection the majority of the mice remain alive for many weeks with an apparently stable pulmonary disease and a steady viable bacillary population in the lungs.

It is tempting to suggest from the results of these investigators that in chronic murine tuberculosis the bacilli remain viable but do not divide, in other words, that the equilibrium is static and not dynamic. Yet evidence based on viable counts alone does not really exclude the possibility that the equilibrium results from a nice balance between the rate at which the tubercle bacilli multiply and the rate at which they are destroyed by the host. Knowledge of the total as well as the viable populations of tubercle baclli present in the tissues should provide a more precise measure of the state of the bacilli in such a chronic infection. We therefore first followed in parallel the viable and the total (stainable) bacillary population in the lungs of mice with chronic tuberculous infection, using the method recently described (Hart and Rees, 1960) for dual counting in tissue homogenates. The same method was then used in similar mice receiving a powerful bactericidal combination of drugs. By disturbing the host-parasite equilibrium in this way it was hoped to follow the fate of the killed bacilli in the lungs.

MATERIALS AND METHODS

Chronic tuberculous infection.—Female mice (18–20 g.) of the albino P strain were infected intravenously with 0·02 mg. wet-weight bacilli (containing about 8×10^6 viable units) of the human virulent strain of *Mycobacterium tuberculosis* H37Rv, prepared by grinding a fully-grown culture (2–3 weeks old) obtained from the surface of Proskauer and Beck's liquid

medium. Under these conditions only about 30 per cent of the mice died from tuberculosis within the first 8 weeks (80 per cent of these deaths, in fact, occurred by the 5th week). The majority of the remaining mice survived in an apparently healthy state for many weeks with an established and predominantly pulmonary infection. Mice killed during this stable phase showed discrete and proliferative-type lesions in the lungs. During the stable phase a few mice died of respiratory insufficiency (with obvious cyanosis) due to cellular infiltration and oedema of the lungs, and not due to progression of the infection with an increase in the number of tubercle bacilli (Sever and Youmans, 1957). This fairly stable infection is similar to the one used for studying the effect of macrocyclon on chronic mouse tuberculosis (Hart and Rees, 1960).

Combined isoniazid-pyrazinamide treatment.—Both drugs were administered in the diet by carefully mixing with powdered diet 41B (Bruce, 1958) to give final concentrations of 0·025 per cent isoniazid and 2 per cent pyrazinamide, representing a daily intake of about 30 μg. and 4·8 mg., respectively, per g. body weight. The mice were given fresh diet daily at the rate of 5 g. per mouse. Treatment was started 60 days after the infection.

Preparation of lung homogenates.—At appropriate intervals 3–6 mice were taken at random from apparently healthy members of the treated and/or untreated group. The treated selected mice had received no drug in the diet for the preceding three days. The mice were killed by exposure to coal-gas, and under sterile conditions the chest wall was opened and both lungs removed. The lungs from each mouse were homogenized individually under aseptic conditions by hand with pestle and mortar and each lung suspended in 10 ml. 0·01 M phosphate buffer pH 7·0.

Number of viable units of tubercle bacilli in lungs.—Viable counts were made by the methods already described by Hart and Rees (1960). Colonies appearing on the plates inoculated with suitable dilutions of each homogenate were counted after incubating the plates at 37° for 14 days in plastic bags.

Total number of acid-fast bacillary units in lungs.—Total bacillary counts (of stainable acid-fast bacilli) were made concurrently with the viable counts on each homogenate of mouse lung by the method recently described by Hart and Rees (1960). The number of stained (acid-fast) bacilli in each lung were determined from counts on spread-smears prepared from a known volume of homogenate. The spread-smears were almost cell-free and the bacilli either single or in small groups. Since a "*viable unit*" count is based on the assumption that a colony can originate from a single bacillus or group of bacilli, the total bacillary counts in this study were brought into line by counting each stained bacillus or group of bacilli as one unit—giving the total acid-fast bacillary unit count.

All counts were expressed as $\log_{10}$ viable, or total acid-fast bacillary, units/organ. Simple averages of these logarithms were used for the charts.

RESULTS

Thirty-seven surviving mice from an original stock of 60 infected (35 per cent having died from tuberculosis) were available on day 59 for this experiment. On day 59 four mice were picked at random and killed to determine the viable and total bacillary units per lung. On day 60 the 33 remaining mice were randomized into 2 groups, untreated (17 mice) and isoniazid-pyrazinamide-treated (16 mice); treatment was from day 60 until day 172. Mice were killed on days 83, 105, 138 and 175, when the experiment was terminated. On days 83 and 105, 3 mice were killed from both the untreated and treated groups; on day 138, 4 from the untreated and 3 from the treated groups; and on day 175, 6 mice from the treated group. Only fit mice were killed, and these were picked each time at random from the 2 groups. Treatment was withheld 3 days before killing, in order to avoid carry over of isoniazid and pyrazinamide in the lung homogenates. The individual and average viable and total bacillary units/lung at each time of killing, expressed as $\log_{10}$, are given in the table, and the average lung counts are also shown in the figure.

The table shows that the total count in each lung from the untreated mice was very similar to, though usually slightly greater than, the viable count; in 11 of the 13 paired figures the total counts slightly exceeded the viable, while in only 2 they were slightly less. (In one of the lung homogenates obtained on day 138 the number of viable units was consistent with the series, but inexplicably the number of stained bacilli was too low for counting.) It may be concluded that there was no build-up of dead bacilli in the lungs, most of the bacterial population in this chronic tuberculous infection being viable. This table, as well as the figure, shows that both viable and total counts from the untreated mice remained remarkably constant throughout the period of 79 days, indicating a steady population.

TABLE.—*Viable and Total Counts of Bacilli in the Lungs of Untreated and Isoniazid-Pyrazinamide Treated Mice in Chronic Tuberculosis*

	Untreated					Isoniazid-pyrazinamide treated*				
Day	Mouse	Viable count (log)	Average viable count (log)	Total count (log)	Average total count (log)	Mouse	Viable count (log)	Average viable count (log)	Total count (log)	Average total count (log)
59	1	6·95	7·38	7·10	7·61	—	—	—	—	—
	2	7·34		7·79		—	—	—	—	—
	3	6·98		7·22		—	—	—	—	—
	4	8·24		8·34		—	—	—	—	—
83	1	6·64	7·30	6·89	7·39	1	6·04	6·18	7·64	7·47
	2	8·56		8·51		2	6·61		7·77	
	3	6·70		6·77		3	5·88		7·00	
105	1	7·45	7·25	7·63	7·53	1	3·18	3·29	6·92	7·13
	2	7·13		7·38		2	4·00		7·71	
	3	7·18		7·57		3	2·70		6·77	
138	1	6·72	6·95	6·89	7·27	1	2·70	2·27	6·39	6·68
	2	6·32		—†		2	2·10		6·62	
	3	6·78		6·98		3	2·00		7·03	
	4	7·98		7·93		—	—	—	—	—
175	—	—	—	—	—	1	< 1·00		6·47	6·35
	—	—	—	—	—	2			6·38	
	—	—	—	—	—	3			5·47	
	—	—	—	—	—	4			7·10	
	—	—	—	—	—	5			6·89	
	—	—	—	—	—	6			5·77	

* Treatment with drugs mixed in diet to give 0·025 per cent isoniazid and 2·0 per cent pyrazinamide, started day 60 and continued to day 172 (inclusive). Treatment withheld 3 days before each killing.

† See text, p. 85.

The table and the figure also show that combined treatment with isoniazid and pyrazinamide had a very powerful killing effect on the tubercle bacilli. After the first 3 weeks of combined treatment the average viable count had already fallen by 95 per cent, and continued to fall steadily throughout the treatment period. In striking contrast the total counts from the treated mice remained high for the first 3 weeks of treatment and then only fell gradually (table). These findings result in an ever-increasing divergence between the viable and total counts from the treated mice, shown in the figure. The high total bacterial count in the

treated mice maintained during the period when the viable bacterial population was greatly diminishing indicates that large numbers of tubercle bacilli killed by combined isoniazid-pyrazinamide treatment were retained in the infected lung. It also follows from the total count (based as it is on stained acid-fast bacilli) that these bacilli retained their gross staining characteristics for many weeks after being

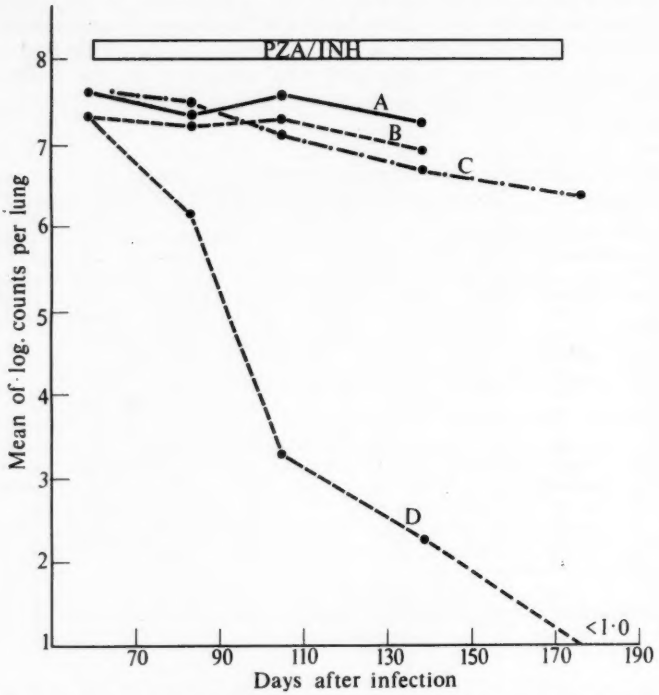

FIG.—Viable and total populations of *Myco. tuberculosis* in mouse lungs during a chronic infection (0·02 mg. H37Rv), and effect of combined treatment with isoniazid-pyrazinamide.

A = untreated, total count.
B = untreated, viable count.
C = PZA/INH, total count.
D = PZA/INH, viable count.

killed by this treatment. The gradual fall in the total count in treated mice may, however, indicate a slow removal of dead bacilli from the lung, a failure of the dead bacilli to stain with carbol fuchsin, or their disintegration.

Similar results were obtained in a second, duplicate, experiment.

DISCUSSION

The trends of the viable and total counts from the lungs of the untreated mice in the present experiments closely resembled those described in the untreated

controls from a study of macrocyclon in chronic murine tuberculosis (Hart and Rees, 1960).

From the closeness of the two counts and their constancy throughout the observation period it was concluded that the majority of the bacilli were viable and not multiplying, but were in a static or resting phase—otherwise, if multiplying and being killed, the total count would rise.

This conclusion was, however, subject to the proviso that dead organisms were not being removed from the lungs (or that their acid-fastness was not being lost). If killing by the host in untreated mice occurred at the same rate as multiplication and the dead bacilli were removed (or their stainability lost), the steady and closely approximating viable and total counts could result.

Treatment with isoniazid-pyrazinamide rapidly produced large numbers of dead bacteria in the lungs, as indicated by the steeply falling viable count. Yet the total count remained steady for 3 weeks, and then only fell gradually. This indicates that a high proportion of the dead organisms remained in the lungs without loss of acid-fastness, and would favour the view that the organisms in the untreated mice were in a static or resting phase rather than in a dynamic equilibrium between multiplication and destruction.

On the basis of a static population of bacilli in the lungs of mice it would be reasonable to find a bactericidal effect with isoniazid and pyrazinamide, since these comprise one of the most powerful drug combinations known against tuberculous infections. On the other hand ineffectiveness of macrocyclon in the chronic infection is attributable to its known bacteriostatic action (Hart and Rees, 1960).

Wallace (1961) has shown that *in vitro*-grown tubercle bacilli in the stationary phase are less easily killed by moderate heat than bacilli in the log phase of growth. It is therefore of interest that this author has found that bacilli obtained from selected mice with chronic tuberculosis in our present series were also less susceptible to heat than bacilli obtained from mice with acute tuberculosis.

SUMMARY

In chronic murine tuberculosis the majority of animals remain alive for many weeks with an apparently stable pulmonary disease and a steady viable bacillary population in the lungs. The latter results suggest that the tubercle bacilli remain viable but do not divide—a "static equilibrium", yet evidence based on viable counts alone does not exclude a "dynamic equilibrium". This equilibrium was therefore analysed further by following in parallel the viable and the total (stainable) bacillary populations in lung homogenates from mice with chronic tuberculosis.

The total counts from the lungs remained steady and were similar to the viable counts from day 60–138 of an untreated chronic infection. The results indicated that unless dead organisms were being removed from the lungs or that their acid-fastness was being lost, the bacillary population was viable and not multiplying.

When similar mice received combined treatment with isoniazid and pyrazinamide, although the viable counts fell steeply throughout the treatment period, the total counts remained high, falling slowly after 3 weeks' treatment below the total counts of the untreated mice. These results indicated that a high proportion

of the dead bacilli (killed by treatment with isoniazid and pyrazinamide) were not removed from the lungs and retained their acid-fastness.

It is concluded therefore that in chronic murine tuberculosis the bacilli are in a static or resting phase rather than in a dynamic equilibrium between multiplication and destruction.

We are indebted to Dr. W. Steenken, Jr., for the H37Rv strain of *Myco. tuberculosis*, and to Mr. C. V. Gibbs for his technical assistance.

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COLLAGEN AS A TENSILE COMPONENT IN THE DEVELOPING CHICK AORTA

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The present study, an extension from previous work on the effect of the lathyrus factor, β-amino-propio-nitrile (BAPN) on the collagenous fibres of the developing chick embryo (Levene and Gross, 1959) is an attempt to define more closely the role of collagen as a tensile factor in the embryo aorta. It is shown that in the developing aorta the collagen concentration gradually increases and that this is accompanied by an increase in the aortic breaking strength ; further, as was previously shown in the case of the entire chick embryo (Levene and Gross, 1959), the capacity to maintain aortic tensile properties depends not only on the quantity, but also on the state of aggregation of the collagen present.

METHODS AND MATERIALS

Fertile eggs of the White Leghorn variety, incubated under constant conditions of temperature and humidity, were killed on successive days from the 10th day of incubation to 5 days after hatching ; the main aortic branches, from the root of the heart to the arch, were carefully dissected from each embryo, a minimum of 20 aortae being pooled for each age group. The specimens were freed from blood, dried to a constant weight *in vacuo* over phosphorous pentoxide in a drying pistol ; aliquots were hydrolysed in a sealed tube with 6N HCl in an oil bath at 140° for 3 hr. and analysed for collagen by the hydroxyproline method of Neuman and Logan (1950). This method is based on the fact that hydroxyproline is unique in animals to collagen, of which it forms approximately 14 per cent dry weight. Each set of comparisons has been performed on a single batch of eggs incubated together ; this is necessary because varying the conditions of incubation can vary the average embryo weight by as much as 30 per cent of the total, and subsequently of the tissue collagen content and tensile properties. Histological preparations were made from formalin-fixed material in the usual way and sections stained with haematoxylin and eosin, Van Gieson, Gomori-Bielschowsky's reticulin and Verhoeff's elastic stain ; sections fixed in cold methanol were examined for metachromasia with toluidine blue.

In order to measure the breaking strength of the main aortic branches the following system, a modification of the one previously described (Levene and Gross, 1959), was devised ; the embryo, freshly removed from the egg, was pinned to a mat, the heart displayed and a loop placed over the root of the heart, and tension applied vertically using a pulley. The most convenient measure of breaking strength was found to be that weight which would sever the aorta within a specific time interval of 30–70 sec. ; it had been previously established on a series of 120 embryos of different ages, that the site of rupture until the 19th day was the aorta itself below the arch and not its origin at the heart.

Lathyrism was induced in the 14-day-old embryo by injection on to the chorio-allantoic membrane, *via* a pin-hole in the shell, of 10 mg. BAPN in 0·1 ml. distilled water. The eggs were re-incubated until the 16th day when they were harvested for breaking strength tests and their aortae collected for histological examination and collagen analysis.

* Research Fellow of the Helen Hay Whitney Foundation.

RESULTS

Normal embryo

Chemical analysis.—The collagen content of the normal aorta (dry weight) gradually rises from 1·45 per cent on the 10th day until it reaches a plateau of 7·5 per cent just prior to hatching (Fig. 1).

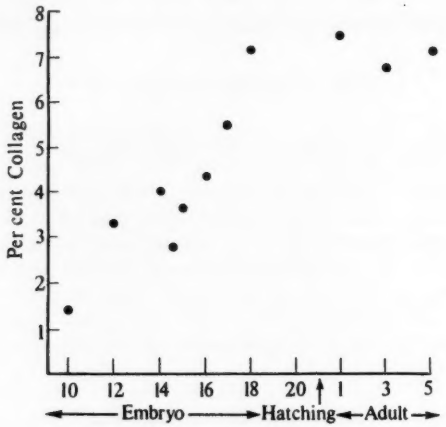


FIG. 1.—Collagen content, on dry weight basis, of normal chick embryo and adult aortae; each point is the average of 3 sets of analyses, performed in duplicate.

Breaking strength.—During development, a direct relationship exists between the weight of the embryo and the load required to sever the aorta in a specific time (Table I). Fig. 2 indicated the direct relationship between collagen content and aortic breaking strength from the 15–18th days inclusive.

TABLE I.—*Comparison at Various Embryonic Ages, of Load Required to Rupture Aorta during a Specified Time Interval*

Embryo age (days)	Weight (average of 15) (g.)	Stretching load required to give average rupture time of 30–70 sec. (average of 15–20 animals) (g.)
14	9·5	7
15	12·6	10
16	14·2	15
17	16·8	20
19	21·5	35

Lathyritic embryo

Histology.—The normal 16-day-old embryo aorta consists of a thick, cellular intima and a media formed of concentric laminae of muscle, elastic fibres and reticulin: the areas in between these laminae are filled with cells and ground

substance which stains metachromatically with toluidine blue stain. At this stage of development the Van Gieson stain does not reveal the presence of collagen in the media. With the stains used it is impossible to differentiate with any certainty between normal and lathyritic aortae (Fig. 3–8).

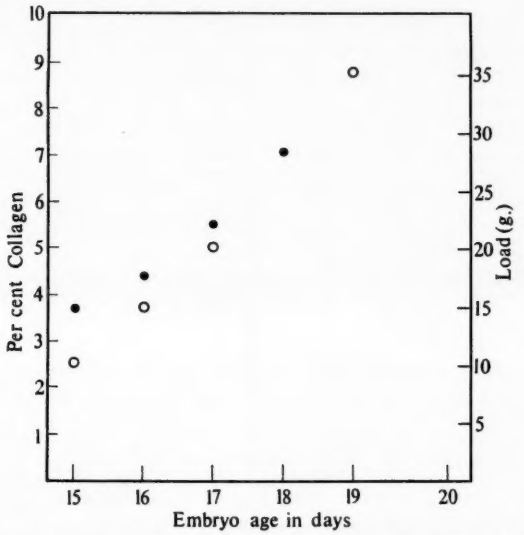


FIG. 2.—Graph to show parallel rise in collagen content and tensile strength of normal embryo aorta from 15th–18th days inclusive.

● Per cent collagen (dry weight).
○ Load required to rupture aorta within 30–70 sec.

Chemical analysis.—Compared with the normal 16-day aorta there is a slight diminution in the amount of collagen present in the lathyritic 16-day aorta (Table II); the latter however possesses a greater collagen content than the normal aorta at 14 days—the time of induction of the disease.

TABLE II.—*Comparison of Hydroxyproline Content and Fragility Indices of Normal 14- and 16-day Embryo Aortae and Lathyritic 16-day Embryo Aortae*

	Average embryo weight (g.)	Per cent hydroxyproline (dryweight)*	Fragility† index
Normal 14-day chick embryo aorta	11·94	1·05±0·02	1·4
Normal 16-day embryo aorta	17·26	1·43±0·15	7·0+
Lathyritic 16-day embryo aorta	16·41	1·31±0·06	0·2

* Average of 3 estimations performed in duplicate.

† Fragility index $= \frac{\text{Time in sec. for aortic rupture}}{\text{Gram body weight}}$ using constant tension of 10 g.

Breaking strength.—In the case of the normal 16-day embryos a direct relationship exists between animal weight and the time required to rupture the aorta

under a constant load; in the case of the lathyritic 16-day animals, however, rupture occurs immediately the load is applied although these animals fall into the same weight group as the normals (Fig. 9).

Extraction of aortic collagen

It has been previously shown (Levene and Gross, 1959) that whereas extraction with cold 1 M saline of skin, bone, tendon and aorta, yields only 2–5 per cent of the total collagen in the normal embryo, the production of lathyrism increases the yield to 20–80 per cent of the total.

Further aliquots of fresh, normal and lathyritic aortae were minced and extracted in the cold for 24 hr. with 3 volumes of 1 M saline, the supernatant spun off in the Spinco preparative ultracentrifuge at 25,000 rpm, their relative viscosities determined in an Ostwald Viscometer at 5°, and the hydroxyproline content measured. The results confirmed previous findings (Table III). The viscosity of the extract and the peak seen in the ultra-centrifugal pattern are due to the presence of collagen as demonstrated by the fall in viscosity and disappearance of the peak after treatment with collagenase (Fig. 10).

TABLE III.—*Comparison of Relative Viscosity and Hydroxyproline Content of* 1 M *Saline Extracts of Normal and Lathyritic Aortae*

	Rel.		Hydroxyproline content (μg./ml.)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Normal 16-day aorta extract	1·3	1·4	20	20
Lathyrus 16-day aorta extract	3·1	3·5	80	123

DISCUSSION

This study demonstrates the gradual increase in aortic collagen which accompanies the growth in the normal chick embryo; this finding is comparable to that of Harkness, Harkness and McDonald (1957) who show an increase in the collagen content of the aortic arch from 8–9 per cent in the puppy to 20 per cent in the dog; similarly in the growing rat the aortic collagen steadily increases from 5 per cent at 3 weeks to 8·1 per cent at 2 yrs. (Kao and McGavack, 1959).

It is also seen that growth is associated with increased ability to withstand experimental stress; this parallel rise in collagen content and stress resistance

EXPLANATION OF PLATE.

FIG. 3.—Histological appearance of normal 16-day-old embryo aortic media (H. and E.) ×390.

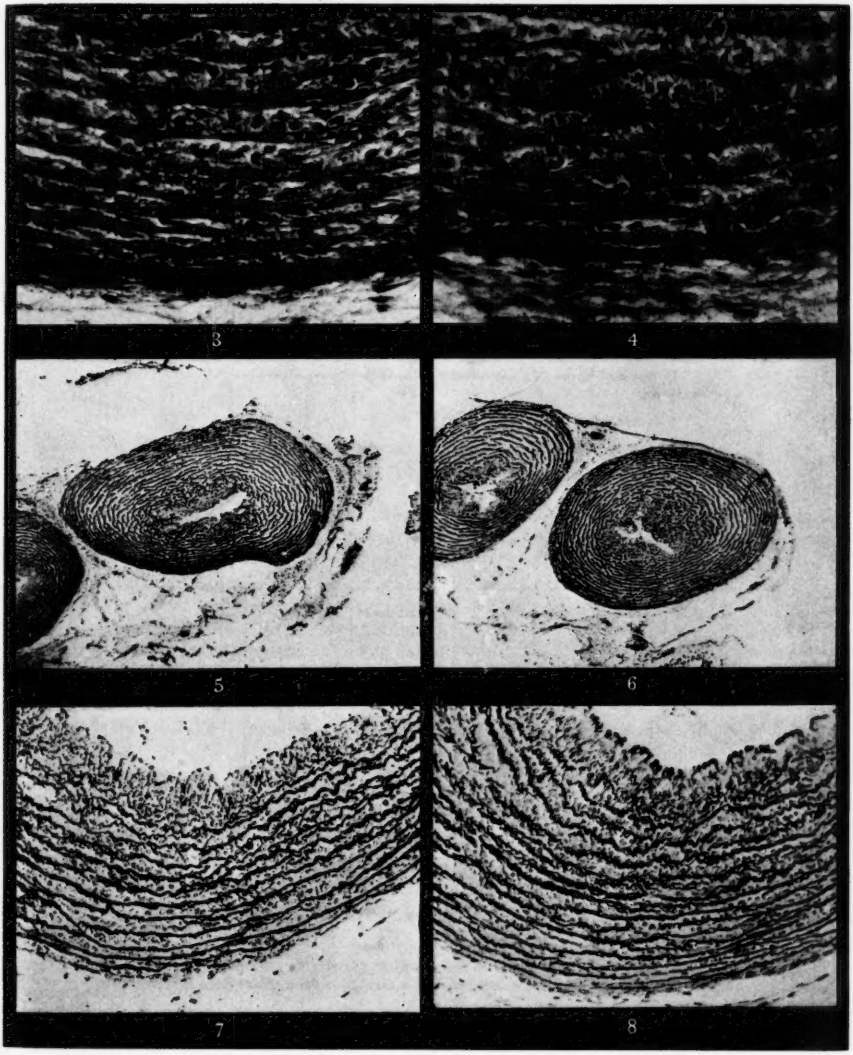
FIG. 4.—Histological appearance of lathyritic 16-day-old embryo aortic media (H. and E.). ×390.

FIG. 5.—Normal 16-day aorta (reticulin). ×36.

FIG. 6.—Lathyritic 16-day aorta (reticulin). ×36.

FIG. 7.—Normal 16-day aorta (Verhoeff's elastic stain). ×190.

FIG. 8.—Lathyritic 16-day aorta (Verhoeff's elastic stain). ×190.



Levene.

is compatible with the view put forward by Burton (1954) that aortic collagen provides maintenance tension against abnormal stress.

The aortic fragility induced by the lathyrus factor is due, not evidently to a diminution in collagen content, but, it is believed, to a loosening of inter-

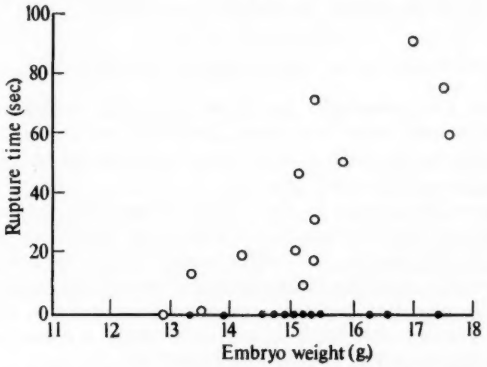
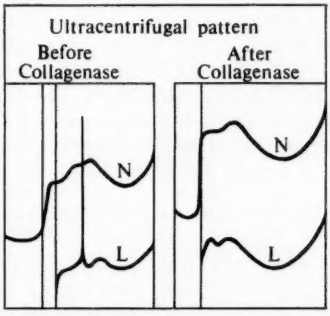


FIG. 9.—Comparison of behaviour under a constant load of 16-day normal and lathyritic aortae.

○ Normal 16-day embryos.
● Lathyritic 16-day embryo (treated 2 days earlier with 10 mg. BAPN).
Constant stretching load = 15 g.

	η rel. Before Collagenase	η rel. After Collagenase	Hydroxy-proline μg./ml.
Norm.	1·3	1·2	20
Lathyritic	3·1	1·3	80



[Reproduced from Levene, C. I. and Gross, J. (1959) *J. exp. Med.*, **110**, 771].

FIG. 10.—Relative viscosity, hydroxyproline content and ultracentrifugal appearance of normal and lathyritic aortic extracts before and after collagenase treatment.

molecular collagen bonding (Levene and Gross, 1959; Van den Hooff, Levene and Gross, 1959) as reflected by the increased collagen extractiblity; changed physical properties of molecular aggregates following change in the state of molecular aggregation have been observed in biological, as well as non-biological polymer systems (Flory, 1956). It appears therefore that in any consideration of tissue tensile properties, it is essential to have information as to the physical

state of the collagen as well as to its quantity. It might be that the phenomenon described by Harkness (1957) in the case of the rat foetal membranes, whose collagen content steadily increases whilst their breaking strength falls rapidy after an initial increase, may prove amenable to such an analysis ; or that such an approach may yield useful information in the study of such conditions as Erdheim's cystic medionecrosis of the aorta, or Marfan's syndrome.

SUMMARY

Analytical data are presented to show that the collagen content of the developing normal chick aorta increases gradually with age ; associated with this there is a rise in the strength of the aorta as measured by the weight required to rupture it during a specific time interval.

The production of lathyrism in the chick embryo by means of the lathyrus factor, β-amino-propio-nitrile, results in a weaker aorta, as measured by the above method. Histologically, no differences from the normal are noted ; chemical analysis reveals a slight diminution in the collagen content of the treated aorta, insufficient to account for the increased fragility ; a comparison of 1 M saline extracts of normal and treated aortae reveals a considerable increase in extractibility of collagen in the treated embryos.

It is concluded that collagen plays a direct role in the maintenance of the tensile strength of the aorta, and that the value of assays for collagen would be enhanced by information as to its functional ability, as well as its morphology and quantity.

Part of this work was done in the Lovett Laboratories of the Massachusetts General Hospital, Boston ; I am deeply indebted to Dr. Jerome Gross for these facilities and for the many helpful discussions. I should also like to thank Professor Sir Howard Florey for his encouragement, Miss Jean McIntosh for her invaluable technical assistance, the Abbott Laboratories, North Chicago for their generous gift of β-amino-propio-nitrile, Miss Christine Court and Mr. Stanley Buckingham for reproductions of graphs and photographs and the Editors of the *Journal of Experimental Medicine* for permission to reproduce Fig. 10.

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INDUCTION OF CELL COMPETENCE TO INFECTION BY POLIOVIRUS RIBONUCLEIC ACID BY TREATMENT WITH MOLAR SODIUM CHLORIDE

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PHENOL extracts of semi-purified poliovirus preparations diluted in molar sodium chloride were reported to infect HeLa cell monolayers, but not to do so when diluted in physiological (Hanks') saline (Alexander, Koch, Mountain and van Damme, 1958). This has been confirmed by experiments reported in this paper. However, the evidence which will be presented indicates that the salt may exert its effect upon the recipient cells, rather than upon the infective agent.

MATERIALS AND METHODS

Cells

ERK cells (Westwood, Macpherson and Titmuss, 1957) were used for all *in vitro* experiments. They were maintained by subculturing cell monolayers at weekly intervals in the usual way, in CSV.6 medium (Cooper, Wilson and Burt, 1959) at 37°.

Virus and nucleic acid

The virus used was the MEF-1 strain of type 2 poliovirus, which had been purified by picking a single plaque from two consecutive cultures. It was grown under single cycle conditions in ERK cells, maintained in suspension in Earle's saline, containing 0·2 per cent glucose; pools obtained in this way regularly contained about 10^9 plaque-forming units (PFU)/ml. From this virus, phenol extracts were prepared at 4° according to a standard procedure (Schuster, Schramm and Zillig, 1956). The absence of any residual virus in the extracts (subsequently referred to as "RNA") was confirmed by specific removal of their infectivity by ribonuclease (10 μg./ml.; 15 min. incubation at room temperature).

Assays of virus and RNA

In vitro assays were performed by a plating method. Monolayers of ERK cells, growing in CSV.6 medium, were prepared in 5 cm. petri dishes. When about 50 per cent confluent they were washed twice with 2·5 ml. volumes of phosphate-buffered saline (PBS) (Dulbecco and Vogt, 1954) lacking calcium and magnesium ions, care being taken to remove as much fluid as possible at each washing. Appropriate dilutions of virus or RNA were inoculated in 0·2 ml. volumes, and allowed to react with the cells for 45 min. at room temperature. A nutrient agar overlay was then added, and the plates incubated at 37° in humidified, 5 per cent carbon dioxide. Three days after incubation a solution of neutral red (iodide; 0·01 per cent in 0·9 per cent sodium chloride) was used to make visible the plaques produced. *In vivo* assays were made by intracerebral inoculation into mice, using 0·03 ml. volumes.

RESULTS

Alexander and her co-workers have noted that poliovirus RNA shows a linear dose/response relationship when plated on HeLa cell monolayers after

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dilution in high molarity sodium chloride (Alexander *et al.*, 1958). However, with ERK cells the number of plaques produced had no apparent relation to the dilution of RNA inoculated (Table I). This was not due to a lack of uniformity among the monolayers used, since, when the virus from which the RNA was prepared was plated under identical conditions the number of plaques was always proportional to the dilution (see also Table I). It is not known why the number of plaques produced by RNA does not follow dilution, although unpublished observations indicate that the condition of the cell monolayer at the time of infection may have an important influence on the result. When monolayers produced from a heavy sowing of cells are inoculated with RNA the number of resulting plaques is less than on monolayers produced in the ordinary way. This difference does not apply when virus is inoculated. In the experiments described here care was taken to ensure that the monolayers appeared uniform and were growing actively. Nevertheless, the non-linear response with RNA obviously makes it impossible to calculate meaningful titres. For this reason, individual RNA plate counts are given in all tables.

Table II shows that the differential effect on the final plaque yield when RNA was diluted in molar salt or PBS was not found when the parent virus was plated under similar conditions. With virus there was no significant difference between diluents.

The *in vitro* results were not reproduced when RNA was inoculated intracerebrally into mice. In this experiment mice were inoculated with identical dilutions of virus or RNA, each calculated to contain approximately 1 LD_{50}/dose,

TABLE I.—*Plaques Produced on ERK Monolayers by RNA Prepared from MEF-1 Poliovirus and by Virus*

	RNA	Virus
Falling two-fold dilutions ↓	1, c, c, 5, 8,	42, 50, 47, 42, 59, 49 (= 289)
	c/2, 5, c/2, c/2, c/2, c/2	27, 26, 23, 24, 31, 24 (= 155)
	c/2, 2, c/2, c/2, 1, c/2	18, 12, 11, 12, 5, 11 (= 69)
	21, c/2, 24, 25, 1, 0	6, 5, 4, 8, 4, 7 (= 34)

c = Confluent. c/2 = Semi-confluent.
Each symbol or number represents the plaque count on one plate.
Diluent = M-NaCl + 0·04 M sodium phosphate, pH 7·2.

TABLE II.—*Effect of Diluent on Titre of RNA and Virus on ERK Monolayers*

Infective agent	Diluent	Dilution	Number of plaques	Titre (PFU/ml.)
RNA	PBS	10^{-1}	0, 0, 0, 0, 0, 0	—
		10^{-2}	0, 0, 0, 0, 0, 0	
		10^{-3}	0, 0, 0, 0, 0, 0, 0	
		10^{-4}	0, 0, 0, 0, 0, 0, 0	
	M-NaCl + 0·04 M-sod. phosph.	10^{-1}	c, 29, c, c, 12, c	—
		10^{-2}	c/2, c/2, c/2, c/2, c/2, 5	
		10^{-3}	17, 3, 7, 7, 5, 1, 1	
		10^{-4}	1, 0, 2, 2, 0, 0, 0	
Virus	PBS	10^{-4}	125, 83, 86, 103, 87, 113	$9·9 \times 10^6$
		10^{-5}	12, 9, 12, 7, 8, 6, 9, 11	
		10^{-6}	2, 2, 1, 1, 2, 1	
	M-NaCl + 0·04 M-sod. phosph.	10^{-5}	24, 8, 17, 13, 18, 16, 10	$1·5 \times 10^7$
		10^{-6}	2, 0, 2, 2, 0, 0	

using PBS or molar sodium chloride as the diluent. Whereas dilution of RNA in PBS totally suppressed infection (as measured by subsequent plaque production) *in vitro* (Table II), this appeared not to be the case when the nucleic acid was inoculated into the mouse brain ; under the latter conditions, PBS appeared at least as efficient a vehicle for RNA infection as molar salt. The response to virus was not affected by the diluent (Table III).

TABLE III.—*Effect of Diluent on Titre of RNA and Virus Inoculated into Mice*

Infective agent	Diluent	Mortality*
RNA	PBS	5/28, 9/28†
	M–NaCl + 0·04 M–sod. phosph.	17/26, 9/29
Virus	PBS	20/25, 14/25
	M–NaCl + 0·04 M–sod. phosph.	20/25, 20/23

* 0·03 ml. volumes, calculated to contain approximately 1 LD_{50}, were inoculated intracerebrally.
† Numerator = number of mice killed.
Denominator = number of mice inoculated.

The evidence presented in Tables II and III suggests that the facilitating effect of high molarity saline *in vitro* is upon the cells themselves rather than upon the nucleic acid. This suggestion was confirmed by the following experiment. ERK cell monolayers were prepared and washed as already described. They were then treated with either PBS or molar sodium chloride for 45 minutes, the fluids removed, and the monolayers washed before inoculation with RNA. The result is shown in Table IV. Clearly, prior treatment of monolayers of ERK cells with high molarity sodium chloride somehow enables them to become infected with RNA diluted in PBS. Although (as shown in Table I) the number of plaques is not proportional to the dilution, the plate counts in Table IV nevertheless indicate that the plaque yield is not as high as on plates in which the RNA is diluted in molar salt.

TABLE IV.—*Effect of Cell Treatment and Diluent on Plaque Production by RNA*

Cells treated with	Diluent	Dilution	Number of plaques
PBS	PBS	10^{-1}	0, 0, 0, 0, 0, 0
		10^{-2}	0, 0, 0, 0, 0, 0
		10^{-3}	0, 0, 0, 0, 0, 0, 0
		10^{-4}	0, 0, 0, 0, 0, 0, 0
PBS	M–NaCl + 0·04 M–sod. phosph.	10^{-1}	c, 29, c, c, 12, c
		10^{-2}	c/2, c/2, c/2, c/2, 5
		10^{-3}	17, 3, 7, 7, 5, 1, 1
		10^{-4}	1, 0, 2, 2, 0, 0, 0
M–NaCl + 0·04 M–sod. phosph.	PBS	10^{-1}	11, 0, 48, 44, 6, c/2
		10^{-2}	0, 3, 4, 0, 2, 0
		10^{-3}	0, 0, 0, 0, 0, 0, 0
		10^{-4}	1, 0, 0, 0, 0, 0, 0

DISCUSSION

The role of molar sodium chloride in influencing infection of ERK cell monolayers with RNA is unknown, but clearly the competence of the cells to react successfully to the nucleic acid is affected. It may be that the high molarity

merely changes the permeability of the cell membrane by a direct osmotic effect: or that a secondary effect is involved, so that the cell in taking up a low ionic strength medium (PBS) to dilute the molar salt-induced intracellular hypertonicity somehow becomes able to initiate production of virus following exposure to RNA. The similarity in the infectivity of RNA for the mouse brain in the two diluents may reflect the fact that *in vivo* the cells are bathed in tissue fluids, so that, although the composition of these fluids may be upset locally and temporarily, the tissue cells would not be in the same environment as they are *in vitro*. The situation may be analogous to that observed with EMC virus (Huppert and Sanders, 1958), in which injection into mice increased the susceptibility of ascites tumour cell populations to infection by virus nucleic acid.

Since this work was done Boeyé (1959) has also reported that infection with poliovirus RNA may be dependent upon the action of hypertonic salt, although he showed that subsequent treatment of RNA-infected cell monolayers with solutions of decreasing salt concentration can actually result in an increase in the final plaque yield. However, Boeyé's complex washing procedure itself may be inducing cell competence, and the RNA diluent may be irrelevant. Nevertheless, with one line of cells (CaIC17), the same number of plaques was consistently obtained either with or without treatment by salt solutions of different strengths, confirming that the action of hypertonic salt is upon the cells rather than upon the nucleic acid.

SUMMARY

RNA prepared from the MEF-1 strain of type 2 poliovirus is able to infect ERK cell monolayers when diluted in molar sodium chloride, but not when diluted in PBS. The infectivity of the virus is the same in either diluent. With RNA, the number of plaques produced is not proportional to the dilution of the inoculum. The different *in vitro* response with the 2 diluents is not paralleled by a similar difference on intracerebral inoculation into mice. This, and further, evidence indicates that the role of molar sodium chloride in influencing infection is upon the ERK cells themselves rather than upon the virus nucleic acid.

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SURVEY OF PAPERS

SHUSTER and CALLAGHAN have examined the generally accepted proposition that in mammalia proteinuria results from failure to reabsorb protein filtered through the glomerulus. Experimental evidence has been produced which suggests that in rats proteinuria may arise as a result of the secretion of protein by renal tubules (p. 1).

BECK tested sera from blood donors and patients for the presence of anti-antibody, which he detected by its capacity to agglutinate human Group O Rh +ve erythrocytes sensitized with incomplete anti-D serum. He found this anti-antibody more commonly in patients than in donors ; it was stable on storage at −16° and resisted heating at 56° for 30 min.; its titre was higher against fresh than against stored erythrocytes ; and its activity was enhanced by a dialysis-labile co-factor present in normal human serum. The anti-antibody could not be absorbed by complexes of other human antibodies with their antigens, nor did it fix complement with its own antigen (p. 7).

WATSON studied the mast cells in the myocardium of rats with hypercholesterolaemia induced by a lipogenic diet.

He found that the morphology and concentration of these mast cells were not impaired by this dietary regime and concluded that the mast cells were probably not involved in the early stages of vascular lipid infiltration (p. 15).

NORTH and HAZEL DOERY incubated ganglioside prepared from ox brain with staphylococcal, tetanus and diphtheria toxins. Provided the incubation period was adequate this neutralized the effect of the first 2 toxins in mice and of the last in guinea-pigs. Heating the ganglioside enhanced its protective effect, and serum albumin inhibited it (p. 23).

STAMP has treated rabbits with staphylococcal vaccines and subsequently showed that they were protected when challenged with a weakly α-toxigenic strain of the organism. He prepared his vaccines from a soluble fraction of the organisms obtained in most cases by ultrasonic disruption. He found no appreciable amounts of α-antitoxin in the immunized animals (p. 30).

BERDJIS has taken 2 groups of 25 male and 25 female Sprague-Dawley rats and given them 25 r total body irradiation. This induced "masculinization" of the submaxillary glands of the female rats resulting in tubular hypertrophy and acidophilic granules in the epithelium.

He concludes that most of these changes were influenced by hormonal imbalance related to ovarian function and that the submaxillary epithelial changes can be used to evaluate ovarian function in rats (p. 38).

JOAN TAYLOR, PATRICIA WILKINS and PAYNE have continued their work on the relation of the rabbit gut reaction to enteropathogenic strains of *Esch. coli*. They have investigated the effects of the numbers of organisms, the colonial form, methods of keeping cultures, media, and immunity upon the reaction (p. 43).

HOBSON and PEARSON prepared purified particle (V) antigens from strains of influenza of the families A, A1 and A2. Finding that the V-antigens fixed complement specifically with homologous ferret antisera, which also specifically inhibited haemagglutination by the allantoic fluids from which the antigens were derived, the authors used the fluids and the antigens to examine 2 batches of paired human sera. The first batch were obtained from volunteers before and after immunization with a killed A2 vaccine, and the others from patients before and after infection. Both HI and V–CF tests revealed a specific response in the vac-

cinees, but patients convalescent sera reacted with all the V antigens, while showing a greater but far from complete specificity in the HI tests. The authors discuss their results in relation to the antigenic structure of influenza viruses (p. 53).

NAKIĆ, SILOBRČIĆ, NAKIĆ and BUNAREVIĆ joined pairs of rats in parabiotic union and simultaneously challenged the right partner with a pedicled full thickness skin graft from the left partner. They found that survival time of the grafts depended to some degree on the duration of parabiotic union, the percentage of tolerant animals (recipients) increased sharply in animals separated on the 5th and 6th post-operative days. The significance of their findings is discussed (p. 63).

HOWARD injected hybrid mice intravenously with spleen cells of a parent strain. This caused a " graft against host " reaction, intensified if the donor had been immunized by a skin-graft from the other parent strain. The reaction was followed by increased reticulo-endothelial (RE) activity in the hybrid, maximal 15 days after receiving spleen cells, and detected by colloidal carbon clearance from the blood. This increased activity was, the author thinks, a response to an accumulation of dead cells at sites of graft-host interaction, and was probably mediated by RE cells in liver sinusoids which could be seen to be increased in number. Spleen enlargement preceded that of the liver, and was due to an increase in the number of plasma cell precursors (p. 72).

REES and HART caused a chronic pulmonary tuberculous infection in 20 g. mice by injecting 0·02 mg. bacilli (H37Rv) intravenously. Total and viable bacterial counts on lungs of surviving mice remained equal and constant from 60–138 days after injection. If such mice were treated with isoniazid and pyrazinamide, viable counts declined rapidly, but total counts only very gradually. The authors conclude that in this chronic murine tuberculosis, the constant bacterial counts depend upon the bacilli being in a static, non-multiplying phase, and are not maintained by a balance between multiplication and destruction (p. 83).

LEVENE showed that the collagen content of the developing normal chick aorta increases with age and this is associated with an increase in strength of the vessel wall as measured by the weight required to produce its rupture.

The production of lathyrism in the chick embryo by means of β-amino-propio-nitrile results in a weaker aorta, but histologically no alteration from the normal; and treated aortae revealed a considerable increase in the extractability of collagen from the treated embryos.

The author concludes that collagen plays a direct role in the maintenance of the tensile strength of the aorta and that the value of assays for collagen would be enhanced by information as to its functional ability as well as its morphology and quantity (p. 89).

HOSKINS has confirmed that HeLa cell monolayers may be infected by phenol extracts of semi-purified poliovirus diluted in molar sodium chloride, but not when diluted in physiological saline. Using ERK cells he has produced evidence that the salt exerts its effect upon the cells rather than upon the virus (p. 95).